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NEUROBIOLOGY OF SOMAN

ANNUAL/FINAL REPORT

Michael T. Shipley, William T. Nickell, Matthew Ennis, Mohamed El-Etri, and Beata R. Frydel

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19. ABSTRACT

This report documents progress in an ongoing study of the central effects of organophosphate (OP) poisoning. In previous work, we have described the anatomy and physiology of a model central cholinergic system, the projection from the horizontal limb of the diagonal band (HDB) to the main olfactory bulb. These basic studies have led to experiments described in the present Report, which focus more directly on the mechanisms of OP-induced seizures.

We have used the proto-oncogene *c-fos*, a marker for neural hyperactivity or cellular stress, to determine the location of early signs of seizure damage after a single sublethal dose of the OP, soman. In rats exhibiting behavioral convulsions, but not in non-convulsive rats, *c-fos* was present in specific layers of the piriform cortex 30 min after injection with soman. At later times, *c-fos* could be found in other cortical structures. Since piriform cortex is the structure suffering the most obvious damage after soman intoxication, this finding suggests that *c-fos* expression is a reliable marker for the early stages of neural damage and that seizures begin in piriform cortex and spread to other brain structures.

Astrocytes have long been known to be involved in the response to injury of the CNS. The importance of these cells in the response to injury has been recently emphasized by the discovery of a marker specific to astrocytes, glial fibrillary acidic protein (GFAP). We have used this marker to demonstrate that astrocytes are activated soon after the onset of seizures.

We also demonstrated significant large decreases in levels of norepinephrine (NE). Thus, we examined the brainstem source neurons of these transmitters for *c-fos* expression following soman intoxication. Only in locus coeruleus (LC), the source of noradrenergic innervation of the forebrain, was *c-fos* expressed. This finding is consistent with evidence that acetylcholinesterase (AChE) inhibition by soman resulted in excess ACh in LC, and therefore, in stimulation of LC neurons.

We tested this hypothesis directly by injecting soman into LC from a micropipette while recording the activity of single LC neurons. Soman injection caused a large (5 X) increase in firing rates of LC neurons. This increased firing rate was completely blocked by systemic injection of the muscarinic antagonist scopolamine.

The involvement of NE in the generation of OP seizures could provide an important new therapeutic approach to soman poisoning. Previous studies have shown that an α_2 adrenergic agonist, clonidine, provides some protection against the convulsive, but not the lethal, actions of soman. Clonidine's protective action was synergistic with the protective actions of atropine. The studies presented here provide a possible mechanistic explanation for this protective effect: clonidine activates inhibitory autoreceptors located on LC neurons, preventing the increase in firing rate which would otherwise follow AChE inhibition. Therefore, clonidine protects target cortical structures from exposure to excess NE.

Thus, since a variety of noradrenergic agonists and antagonists are already in clinical use, further studies of these agents in combination with cholinergic antagonists could yield an improved postexposure therapeutic regimen which could be rapidly deployed to military personnel.

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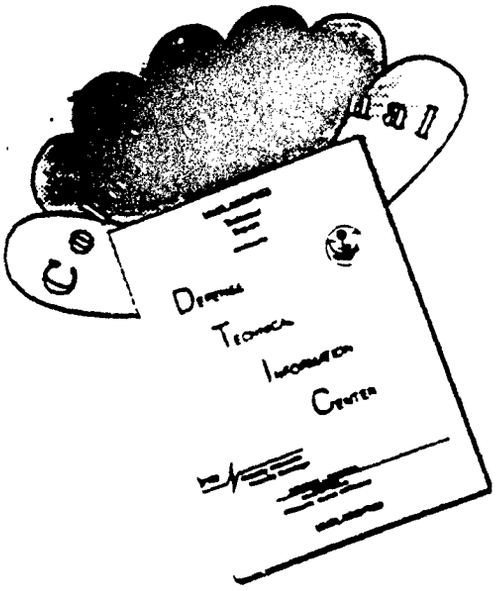

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FOREWORD

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources (DHHS Publication No. (NIH) 86-23, Revised 1985).

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1. INTRODUCTION

This report documents progress in an ongoing study of the central effects of organophosphate (OP) poisoning. In previous work, we have described the anatomy and physiology of a model central cholinergic system, the projection from the horizontal limb of the diagonal band (NDB) to the main olfactory bulb. These basic studies have led to experiments described in the present report, which focus more directly on the mechanisms of OP-induced seizures.

A major problem in treatment of OP intoxication is that drugs which alleviate peripheral symptoms may not prevent centrally generated seizures which can lead to permanent brain damage. We describe the application of recently developed techniques to address the question of the initial location and neural mechanisms of these seizures. These results lead to the hypothesis that excess release of norepinephrine (NE), caused by cholinergic hyperstimulation of NE source neurons, contributes to the generation of OP-induced seizures. If true, treatment of these seizures might be markedly improved by pharmacological agents directed at NE receptors. Since many such agents are already in clinical use for treatment of cardiovascular and psychological symptoms, effective agents could be rapidly applied clinically without extensive testing.

The proto-oncogene *c-fos* has recently been used by numerous investigators as a marker for neural hyperactivity or cellular stress. We have used this marker to determine the location of the earliest signs of seizure damage after a single sublethal dose of the OP soman (Chapter 2). In rats exhibiting behavioral convulsions, but not in nonconvulsive rats, *c-fos* was present in specific layers of the piriform cortex (PC) 30 min after injection with soman. At later times, *c-fos* could be found in other cortical structures. Since piriform cortex is the structure suffering the most obvious damage after soman intoxication, this finding suggests that *c-fos* expression is a reliable marker for the early stages of neural damage and that seizures begin in piriform cortex and spread to other brain structures.

Astrocytes have long been known to be involved in the response to injury of the central nervous system (CNS). The importance of these cells in the response to injury has been recently emphasized by the discovery of a marker specific to astrocytes, glial fibrillary acidic protein (GFAP). We have use this marker to demonstrate that astrocytes are activated soon after the onset of seizures (Chapter 3).

Our previous studies (Shipley *et al.*, 1990) had demonstrated significant changes in levels of NE and of metabolites of serotonin (5HT) and dopamine (DA) after soman intoxication. Thus, we examined the brainstem source neurons of these transmitters for *c-fos* expression after soman intoxication. Only in locus coeruleus (LC), the source of noradrenergic innervation of the forebrain,

was *c-fos* expressed. This finding is consistent with a variety of evidence that LC neurons are stimulated by acetylcholine (ACh). Thus, it was likely that acetylcholinesterase (AChE) inhibition by soman resulted in excess ACh in LC, and, therefore, in stimulation of LC neurons.

We tested this hypothesis directly by injecting soman into LC from a micropipette while recording the activity of single LC neurons (Chapter 4). As expected, soman injection resulted in a large (mean 5 X) increase in firing rates of LC neurons. This increased firing rate was completely blocked by systemic injection of the muscarinic antagonist scopolamine.

We have extended our studies (Shipley *et al.*, 1988) of changes in monoamine levels in soman-intoxicated rats to additional time points (Chapter 5). These new studies confirm and extend our previous conclusion that OP-induced convulsions are associated with profound depression of forebrain NE. In behaviorally convulsive rats, NE levels were decreased to 32% of NE levels in control rats. However, in rats which received the same dose of soman but did not exhibit behavioral convulsions, NE levels were not different from control rats.

These studies suggest the following hypothesis for the generation of seizures by OP intoxication. AChE inhibition by the OP results in excess ACh in both the piriform cortex and the locus coeruleus. The ACh excess in LC causes rapid firing of LC neurons, with resulting excess NE in piriform cortex. The combination of excess ACh and NE may result in extreme excitability of neurons in piriform cortex, with the result that the neuronal system breaks into seizures. The rapid firing of LC neurons may eventually result in metabolic failure and a decrease in NE release; however, by this time mechanisms involving excitatory amino acids (EAAs) may have strengthened synaptic connections within piriform cortex sufficiently that the cholinergic and noradrenergic hyperstimulation is no longer necessary (Shipley and Nickell, 1989; Chapter 7).

The involvement of NE in the generation of OP seizures could provide an important new therapeutic approach. Previous studies (Aronstam *et al.*, 1986; Buccafusco and Aronstam, 1986; Buccafusco *et al.*, 1988) have shown that an a_2-adrenergic agonist, clonidine, provides some protection against the convulsive, but not the lethal, actions of soman. Clonidine's protective action was synergistic with the protective actions of atropine. The studies presented here provide a possible mechanistic explanation for this protective effect: Clonidine activates inhibitory autoreceptors located on LC neurons, preventing the increase in firing rate which would otherwise follow AChE inhibition. Therefore, clonidine protects target cortical structures from exposure to excess NE.

Thus, since a variety of noradrenergic agonists and antagonists are already in clinical use,

further studies of these agents in combination with cholinergic antagonists could yield an improved post exposure therapeutic regimen which could be rapidly deployed to military personnel.

We have also studied the role of ACh in regulating synaptic actions in a model cortical structure, the olfactory bulb. We previously showed that electrical stimulation of cholinergic neurons projecting to the olfactory bulb from the basal forebrain caused profound potentiation of synaptic responses in the olfactory bulb (Nickell and Shipley, 1988b). We later showed that stimulation of these cholinergic neurons causes a profound inhibition of synaptic transmission in the commissural connection between the two olfactory bulbs (Chapter 6). These observations emphasize the complexity of central cholinergic actions.

2. SOMAN-INDUCED SEIZURES AND PROTO-ONCOGENE EXPRESSION

Introduction

A wealth of recent evidence has demonstrated that depolarization and/or second-messenger activation in brain neurons leads to the rapid induction of proto-oncogenes, the DNA transcriptional regulatory proteins *c-fos* and *c-jun*. After sustained depolarizing and/or second-messenger activation, a series of intracellular messengers cause the rapid transcription of a set of regulatory genes--proto-oncogenes or "early immediate" genes--leading to the rapid synthesis of proto-oncogene protein products. These proteins act on the genome to promote the transcription of additional genes encoding proteins required to maintain the metabolic or physiologic activities of the cell (Bartel *et al.*,1989; Ceccatelli, *et al.*, 1989; Greenberg *et al.*, 1986; Herrera and Robertson, 1990; Hunt *et al.*, 1987; Morgan and Curran, 1986; Morgan *et al.*, 1987; Sagar *et al.*, 1988; Sheng and Greenberg, 1990; Sheng *et al.*, 1990). Thus, induction of proto-oncogenes such as *c-fos* indicates that the cell is adaptively responding to external stimuli by the production of proteins necessary to the continued function of the cell. In addition, under extreme stress proto-oncogenes may also promote the transcription of genes encoding proteins critical to cell survival. Proto-oncogene induction, thus, is the earliest known macromolecular signal of physiological activation of neurons. If *c-fos* expression could be detected *in situ* after soman poisoning, then it might be possible to determine the earliest neurons activated by this OP. Thus, we tested whether soman-induced seizures increased *c-fos* expression.

Methods

Rats were injected (i.m.) with a single dose (77.7 µg/kg) of soman. In previous experiments, this dose has been found to produce convulsions in about one half of injected animals and nearly complete inhibition of acetylcholinesterase (AChE) in target structures. Rats survived 30-45 min, 1 hr, 2 hr, 4 hr, 8 hr, and 24 hr after injection. A minimum of five animals was included in each group.

At the end of the specified survival time, rats were deeply anesthetized with pentobarbital and perfused intracardially with saline followed by fixative. A control, non-injected, animal was perfused at the same time as each experimental animal. Brains were removed from the skull, sectioned at 30 µm, and processed for immunocytochemical identification of *c-fos* according to standard protocols.

Results

C-fos protein was detected by immunocytochemistry as early as 30 min after a single systemic

(77.7 μg/kg) dose of soman (Figure 1). The earliest forebrain neurons to express the oncogene are located in the piriform cortex. By 2-24 hr after soman administration, additional neurons in a variety of cortical and subcortical structures begin to express *c-fos*. The anatomical distribution of this time-dependent wave of *c-fos* expression matches very well to the progressive recruitment of brain structures in the seizures and brain damage caused by soman.

Role of Cholinergic Innervation of PC from Basal Forebrain. Piriform cortex (PC) receives a massive cholinergic innervation from basal forebrain cholinergic neurons in the nucleus of the diagonal band (NDB). We have labelled cholinergic fibers in the PC with antibodies to choline acetyltransferase (ChAT), the synthetic enzyme for ACh; ChAT immunocytochemical staining is definitive of cholinergic innervation. The results demonstrate that the cholinergic innervation of PC is heavier than the corresponding cholinergic innervation of hippocampus and neocortex. The cholinergic innervation is particularly dense in layers I, II, and III; these same cortical layers exhibit dense acetylcholinesterase (AChE) staining. Cholinergic inputs to PC arise in the basal forebrain from the nucleus of the horizontal diagonal band (NDB). Following systemic injections of convulsive doses of soman, rapid (30-45 min) *c-fos* expression is restricted to neurons whose cell bodies and dendrites occupy layers I, II, and III. Therefore, the first neurons in the brain to express *c-fos* after soman administration are neurons that receive the heaviest cholinergic projection of any cortical structure.

A parsimonious explanation for soman-induced seizures and expression of *c-fos* in PC neurons, therefore, is that inhibition of AChE leads to "cholinergic hyperstimulation" of layer II and layer III PC neurons. If this is true, sustained electrical stimulation of the cholinergic neurons in NDB that project to PC should cause endogenous cholinergic hyperactivation. This, in turn, should induce the same pattern of *c-fos* expression in PC as soman. Thus, we tested whether electrical stimulation of NDB induced *c-fos*.

In rats anesthetized with methoxyflurane, intermittent trains of shocks (160 shocks at 10 Hz; 15 sec on - 15 sec off) were applied for 45 min via an intracranial stimulation electrode placed unilaterally in NDB on one side of the brain. At the end of the 45-min period of stimulation, the animals were sacrificed and processed for *c-fos* immunocytochemistry. The results of this experiment strongly confirmed our hypothesis: *c-fos* expression was elevated in layer II and layer III neurons in PC, ipsilateral to the stimulation site in NDB (Figure 2). In the contralateral PC, which does not receive projections from the stimulated cholinergic neurons, there was no *c-fos* expression. Thus, activation of the cholinergic neurons that project to PC, causing sustained endogenous cholinergic hyperstimulation, mimics the pattern of *c-fos* induction caused by systemically administered soman. This result supports the hypothesis that the induction of *c-fos* and the initiation of soman-induced cortical seizures is

triggered by cholinergic overstimulation caused by soman's anticholinesterase action. This hypothetical mechanism we refer to as the "cholinergic trigger".

We further hypothesize that the primary consequence of cholinergic hyperstimulation of PC neurons is to dramatically increase the responsiveness of these cortical neurons to EAAs that are being tonically released by the majority of synapses impinging on them. There is a well-established cellular mechanism by which ACh increases the excitability of cortical neurons to EAAs: ACh, acting via muscarinic receptor-mediated second-messenger systems, blocks a voltage-dependent Ca^{++}-dependent, K^{+}-conductance which causes a hyperpolarization that normally prevents cortical neurons from being overexcited by EAAs. In the presence of sustained, excess ACh, this protective mechanism is inactivated. We propose the hypothesis that during the chronic AChE inhibition caused by soman, excess ACh blocks this protective mechanism with the result that cortical neurons are driven to seizures by tonically active EAA synaptic inputs. Because these cortical neurons are progressively excited by EAA inputs, they release more EAAs at their own synaptic terminals on other cortical neurons, thus further feeding an EAA "chain reaction".

Role of Noradrenergic Innervation from Locus Coeruleus. Our studies of *c-fos* expression after soman showed that the earliest site in the brain to express the oncogene was PC. This was a very telling observation because more than a decade of research had pinpointed PC as the site most vulnerable to soman and had shown that seizures are initiated in this temporal lobe region. However, the rapid spread of seizures to the rest of the cerebral cortex and to subcortical areas suggests that other neural systems might be involved in the genesis and/or propagation of seizures. While the list of other candidate neural systems is potentially very long, there are several candidates which are particularly suspect. One obvious possibility is that soman, either via its cholinolytic actions or some novel mechanism, might have an effect on γ-amino butyric acid (GABA)ergic systems. Picrotoxin, which antagonizes GABA receptors, is a potent seizurogenic compound. Thus, we have examined the possibility that soman might influence GABA neurons. However, the pattern of *c-fos* expression failed to support this working hypothesis; there was no systematic relationship between the pattern or time course of expression of *c-fos* and the known distribution of GABA neurons or GABAceptive brain regions. This, taken with previous evidence that there are no significant neurochemical changes of GABA-related molecules in the first hour or so after soman when seizures are initiated, suggests that GABA systems may not be a primary target of soman's seizurogenic actions.

Another set of candidate neural systems are the brainstem monoamine groups. These systems, exemplified by the nucleus locus coeruleus (LC) and the raphe nuclei, are unique neural systems for several reasons. First, neurons in LC and the raphe nuclei project more globally and diffusely

throughout the CNS than any other neurons in the brain. Thus, by virtue of their massive, global, influence over wide areas of the CNS, these systems are uniquely positioned to exert immediate, widespread changes in neural function. Second, both of these neural systems use monoaminergic neurotransmitters. Neurons of LC synthesize and release norepinephrine (NE); LC is the sole source of NE innervation of the cerebral cortex (including PC) and most other parts of the forebrain (Fallon and Moore, 1978; Foote *et al*, 1983). Neurons in the dorsal and median raphe nuclei synthesize and release either serotonin (5HT) or dopamine (DA). The raphe nuclei are the sole source of 5HT input to the cerebral cortex and most of the forebrain. DA neurons in these two raphe nuclei, along with some DA neurons in the ventral tegmentum area near the median raphe, provide the sole source of DA input to the cerebral cortex. All three of these monoamines (NE, 5HT, and DA) function as "modulatory" transmitters, i.e., their major influence on postsynaptic neurons is to modulate the responsivity of the neurons to the coactivation of other synaptic inputs, usually an EAA input. Thus, the monoamine systems are positioned to simultaneously influence the entire cortical mantle, and they are known to modulate the responsiveness of cortical neurons to co-activation of excitatory amino acid inputs.

Based on these considerations, we conducted a series of experiments to determine whether any of these subcortical monoamine systems showed *c-fos* expression that coincided with the onset of soman-induced seizures. In all animals (n=5) examined at 30 and 45 min after soman, there was little consistent expression of *c-fos* in the dorsal and median raphe and adjacent ventral tegmental area. By contrast, in all animals in which soman induced convulsions, there were high levels of *c-fos* in neurons of the LC (Figure 3). Thus, along with PC, LC neurons are the earliest in the brain to show *c-fos* expression.

If *c-fos* expression in LC neurons signifies that these neurons are hyperactive, then it would follow that very early after soman exposure, there may be an increase of norepinephrine release throughout the forebrain. This, in turn, could potentiate the initiation, the intensity, or the widespread propagation of seizures. Studies in the brain slice have shown that NE acting through a ß- and, possibly, also an a-adrenergic receptor specifically attenuates a K^+-mediated hyperpolarization potential which normally follows the rapid depolarization and spike initiation caused by EAAs. This K^+-mediated afterhyperpolarization potential (AHP) normally functions to limit the duration of the depolarization and action potentials caused by EAAs. The AHP, then, may be a kind of natural brake to overexcitation by EAAs and would function to inhibit seizures. In the hippocampus and PC, NE blocks the AHP, thus causing cortical neurons to become hyper-responsive to EAAs (Collins *et al*, 1984; Gray and Johnston, 1987; Haas and Konnerth, 1983; Madison and Nicoll, 1982, 1986; Nicoll, 1988; Segal, 1981).

If, as suggested by the *c-fos* results, LC neurons are driven to hyperactivity by soman, causing increased release of NE, then the AHP should be significantly reduced or blocked, thus rendering cortical neurons hyper-responsive to EAAs. In this case, NE and ACh hyperstimulation may act cooperatively, or even synergistically, to potentiate cortical neurons' hyper-responsiveness to EAAs because both NE and ACh block the K^+-mediated AHP (Benardo and Prince, 1982; Brown and Adams, 1980; Cole and Nicoll, 1984; Dodd *et al.*, 1981; Dutar and Nicoll, 1988; Halliwell and Adams, 1982; Madison *et al.*, 1987; Nicoll, 1988; Worley *et al.*, 1987). Thus, if soman causes sustained hyperactivity of LC with sustained release of NE, then cortical neurons will be confronted with a "double whammy": Hypercholinergic and hypernoradrenergic stimulation blocking the AHP, thus making cortical neurons hyperactive to EAA synaptic inputs.

Figure 1. Rapid, Selective Induction of *c-fos* and Glial Fibrillary Acidic Protein (GFAP) In Piriform Cortex (PC) by a Single Convulsive Dose of Soman.

Animals were given a single dose (77.7 µg/kg, i.m.) of soman. Convulsing animals were sacrificed at different time intervals from 0 to 120 min (times indicated in middle row). Sections from all animals were processed simultaneously in the same reaction solutions (same antibody concentration), for immunohistochemical staining of *c-fos* (first two columns A, B, D, E, G, H, J, K) or GFAP to stain for reactive astrocytes (third column; C, F, I, L). *C-fos* and GFAP stained sections are serially adjacent.

For the *c-fos* stained sections, low- (first column) and high- (middle column) power micrographs demonstrate localization of *c-fos* staining in layers II and III of PC. Both the number of cells and the intensity of staining increase progressively between 0 (A,B) and 120 (J, K) min. GFAP staining (right column) is visible at 1 hr and is strongly expressed at 2 hr following intoxication. GFAP is located in the same layers as *c-fos*.

GFAP: Glial fibrillary acidic protein. LOT: lateral olfactory tract.

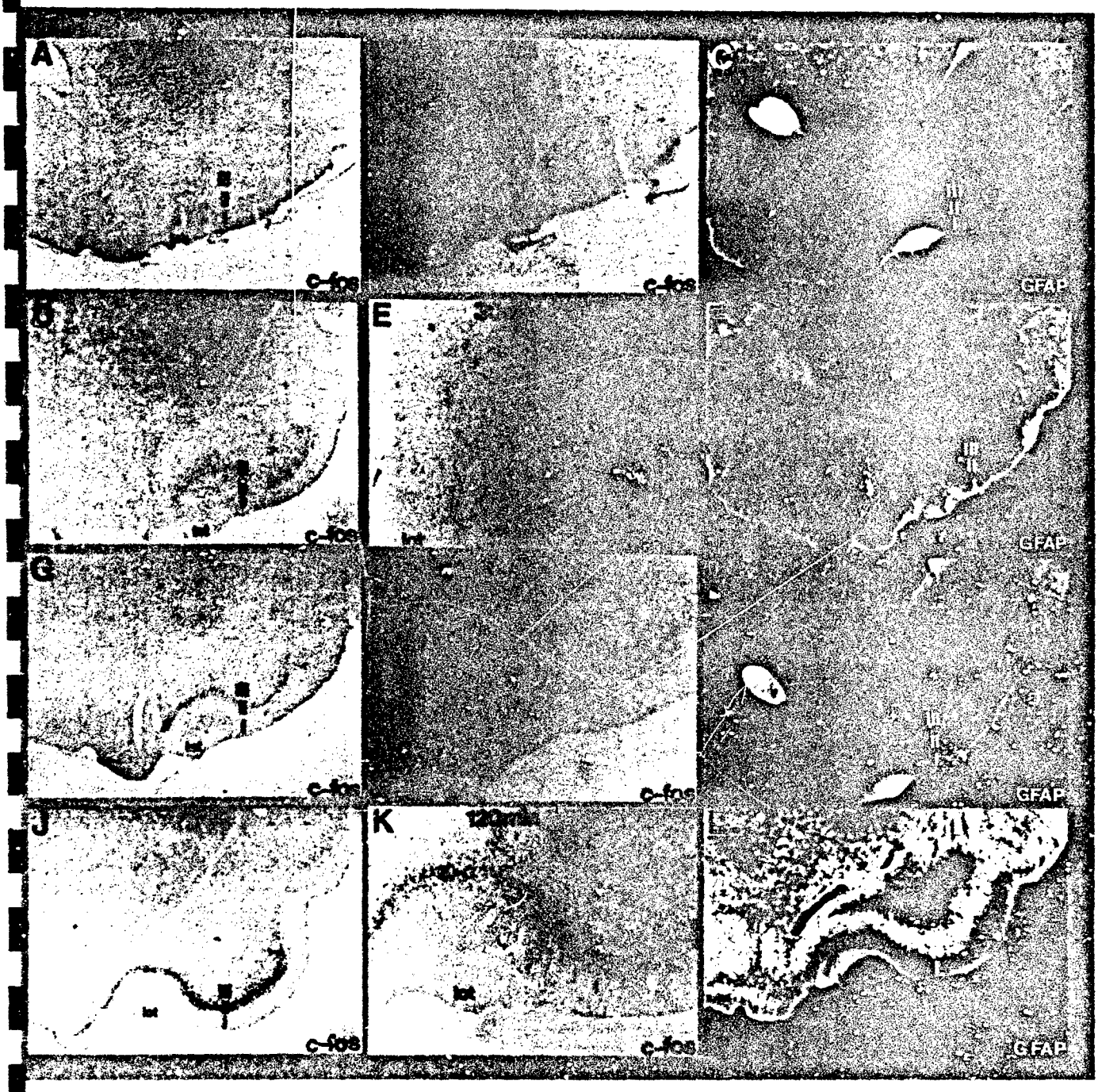
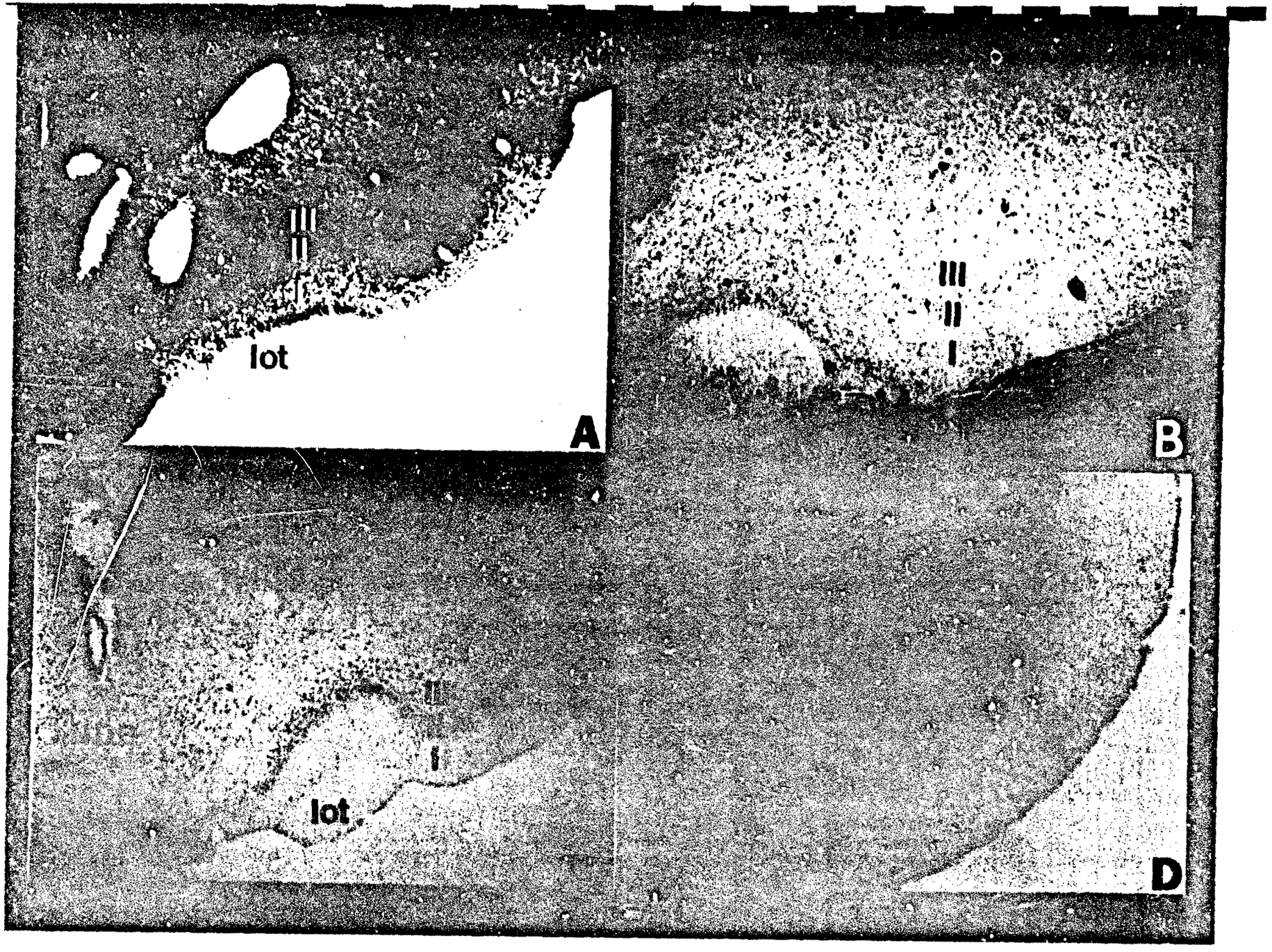

A
c-fos
C
GFAP
E
Int
GFAP
G
Int
c-fos
GFAP
J
Int
K
120mM
Int
c-fos
GFAP

Figure 2. Stimulation of Endogenous Acetylcholine (ACh) Release Induces *c-fos* in the Same Piriform Cortex (PC) Neurons (Layers II and III) as Does Systemic Soman.

A: Acetylcholinesterase (AChE) histochemical staining in piriform cortex (PC) shows that AChE fiber staining is selectively intense in layers II and III, the same layers that express *c-fos* after systemic soman (Figure 1) and after stimulation of the cholinergic input to PC (C and D). LOT = lateral olfactory tract.

B: Choline acetyltransferase (ChAT) immunohistochemical staining of cholinergic fibers innervating PC (darkfield micrograph). Cholinergic terminal staining corresponds to AChE staining and is preferentially heavy in layers II and III.

C and D: Stimulation of cholinergic neurons in the nucleus of the diagonal band (NDB) for 45 min (see text for stimulation parameters) activates *c-fos* in layers II and III in PC, the same layers expressing *c-fos* after systemic soman. In C, part of the stimulation electrode track can be seen at the far left. D shows the *c-fos* expression in layer II and III PC neurons at higher magnification. LOT = lateral olfactory tract.


III
II
I
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D

Figure 3. Rapid, Selective Induction of *c-fos* in Nucleus Locus Coeruleus (LC) Neurons After a Single Convulsive Dose of Soman.

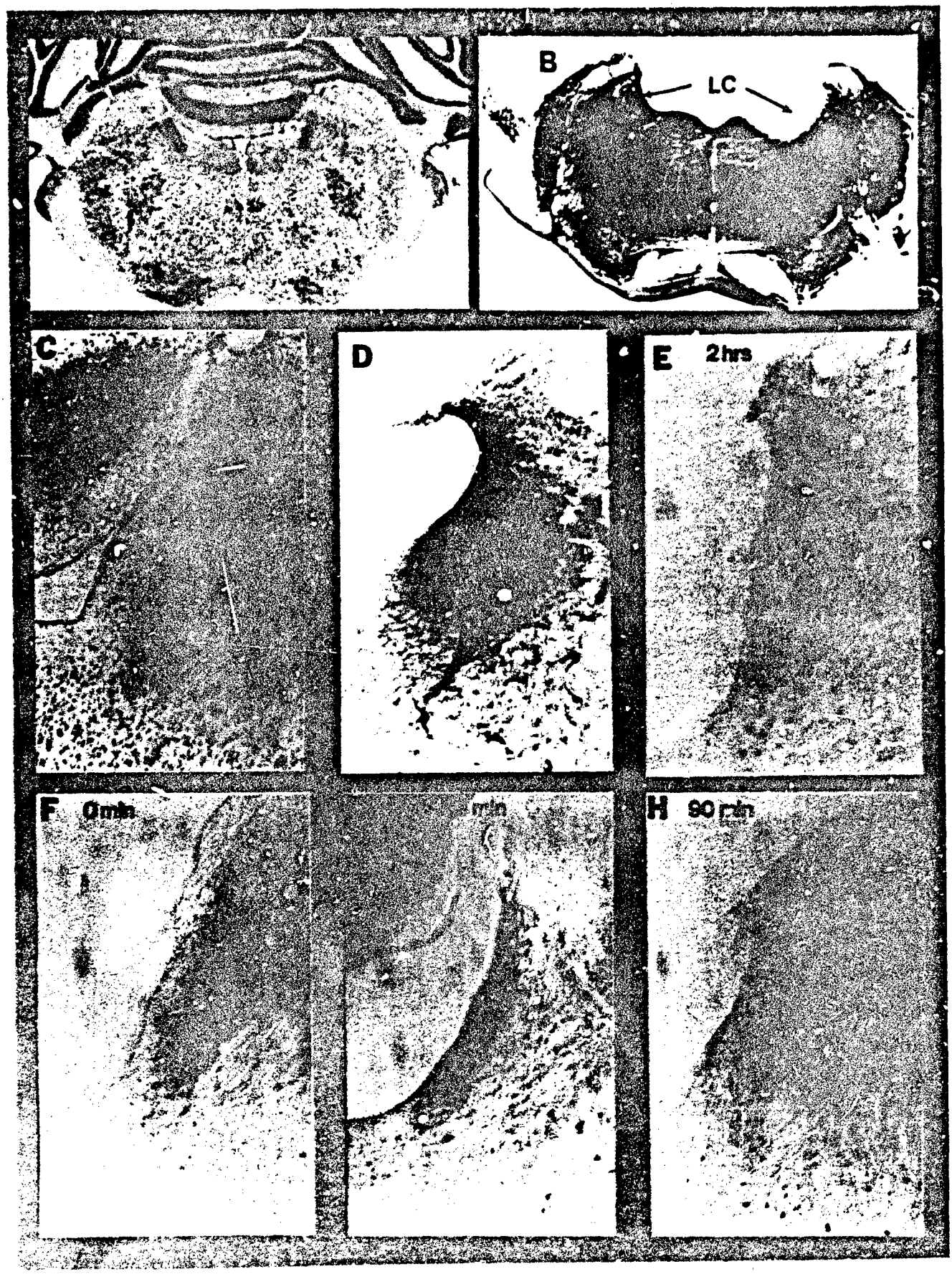
A: Low-power micrograph showing the location of LC. In Nissl-stained material, LC forms a compact nucleus lying along the lateral margin of the IVth Ventricle in the dorsolateral pons.

B: Acetylcholinesterase (AChE)-stained section adjacent to the section shown in A. Note dense AChE staining in LC in the left side (at arrow). On the right side (at arrow), a focal microinjection of soman completely inhibited AChE in LC.

C: Higher-power micrograph of LC from the Nissl-stained section shown in A. Note that LC neurons are densely stained for Nissl substance and form a discrete, compact nucleus.

D: Dopamine-ß-hydroxylase (DBH) immunohistochemical staining of LC neurons. DBH is the key biosynthetic enzyme for norepinephrine. This enzyme is highly abundant in all LC neurons.

E-H: A single convulsive dose of systemically administered soman induces *c-fos* expression in LC neurons. This experiment is similar to that depicted in Figure 1. A group of animals received a single dose of systemically administered soman (77.7 µg/kg). The animals were observed for convulsions. At various time intervals (F = 0 min, G = 45 min, H = 90 min, E = 2 hr) the animals were sacrificed and processed for immunohistochemical detection of *c-fos*. Sections from all animals were processed at the same time and in the same reaction solution to control for variation in processing. At 0 min (F) there is no detectable *c-fos* staining in LC. At 45 (G), 90 (H), and 120 (E) min after soman, there was a progressive increase in the numbers of LC neurons expressing *c-fos*, and the intensity of *c-fos* staining.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H 90 min

Conclusion

The demonstration that piriform cortex (PC) was invariably the first structure in the brain to express *c-fos* after soman-induced convulsions was a pivotal observation. It has long been known that PC has a preferentially low threshold for soman-induced seizures. Of equal importance, it is also well established that the PC is the most vulnerable site in the brain to soman-induced neuropathology. The demonstration that *c-fos* is rapidly induced, specifically in PC neurons, therefore indicates that the expression of this molecule selectively pinpoints the earliest neurons known to be affected by soman. Further, a subset of PC neurons express *c-fos*: The neurons of layers II and III of PC. This selectivity of neuronal subpopulations further reinforced the idea that *c-fos* is pinpointing the neurons affected by soman: Evidence from this and many other laboratories shows that soman-induced cell death is highly preferential for these same layer II-III PC neurons (Wall *et al.*, 1990; Switzer *et al.*, 1990). Thus, after soman, the first neurons in the brain to express *c-fos* are the same neurons that show the highest frequency of cell death in soman-induced neuropathology. *C-fos*, therefore, selectively and sensitively identifies the first brain neurons affected by soman. Animals subjected to the same doses of soman, but allowed to survive for progressively longer times before staining for *c-fos*, showed progressive induction of *c-fos* in other brain regions.

These studies demonstrate that the time-dependent wave of *c-fos* expression mirrors the known time-dependent spread of seizures in soman-exposed animals. Thus, PC neurons express *c-fos* by 30 min; after 45-60 min *c-fos* induction is detectable in hippocampal and some neocortical neurons; and, by 60-120 min *c-fos* is detected throughout most of the cerebral cortex and in some subcortical structures. The laminar pattern of *c-fos* expression in cortex is also quite stereotyped. Throughout most cortical areas, the first neurons to express *c-fos* are the neurons of layers II and III followed later by the neurons of layers V and VI. The significance of this is that neurons of layers II and III are those that receive and give rise to the majority of the corticocortical association connections that anatomically link different areas of the cortex. That *c-fos* is initially expressed in these "associative" layers strongly suggests that the spread of seizures is mediated to a large degree by corticocortical circuits. These circuits predominantly use excitatory amino acid (EAA) transmitters.

Considerable evidence, particularly in the last 5 years, has spotlighted PC as the site in the cerebral cortex most selectively vulnerable to the seizurogenic actions of a number of neuroactive compounds. Of even greater significance from the perspective of soman's seizurogenic action, is that several recent studies have demonstrated that PC is not only selectively seizure-prone, but that seizures initiated in PC spread to the rest of the cerebral cortex. A recent study has even suggested that a rostral subcomponent of PC is especially epileptogenic (Piredda and Gale, 1985); microinjections of the GABA

agonist muscimol into this restricted part of rostral PC can both effectively block the initiation of seizure, and, if given after the initiation of seizures, prevent the spread of seizures to the rest of the cortex. Meldrum and colleagues (Millan *et al.*, 1988) more recently reported that microinjections of EAA agonists at any rostrocaudal site of PC initiates seizures that spread throughout the cortex. In addition, they have demonstrated that femptomolar concentrations of EAA agonists injected into PC can trigger seizures in animals given subconvulsive doses of the cholinergic muscarinic agonist pilocarpine. Meldrum and co-workers have also shown that 1-10 fmol of an N-methyl-D-aspartate (NMDA) antagonist in PC prevents seizures normally caused by convulsive doses of pilocarpine. These findings, taken together with the known pathological vulnerability of PC to soman and our new results showing that PC is the first brain structure to express *c-fos* after soman intoxication, strongly support the hypothesis that soman-induced seizures are initiated in PC and that PC serves as a focus for the propagation of seizures to other cortical areas via EAA-mediated corticocortical association pathways.

The idea that NE release in forebrain may contribute to seizures has precedent in the literature. *In vitro* cellular electrophysiological studies demonstrate that NE acting at a ß-receptor blocks the so-called afterhyperpolarization potential (AHP) in hippocampal pyramidal neurons. As a result of blocking the AHP, these cortical neurons become hyperexcitable to activation of afferent EAA synapses and to exogenously applied EAA agonists. Thus, NE activation of ß-receptors has a seizure-promoting action very similar to that produced by ACh acting at muscarinic receptors. In this regard, it is significant that pharmacologic experiments have demonstrated that ß-receptors are densely concentrated in layer II and layer III of PC and layers II-III of the neocortex (Booze *et al.*, 1989; Jones *et al.*, 1985ab; Palacios and Kuhar, 1980). These are the same layers where we found *c-fos* induction in neurons after soman-induced seizures. Other evidence also points to the possible involvement of NE in soman-induced seizures. Levitt and Noebels (1981) reported that in a single gene seizure-prone mutant mouse, there is a 50% increase in the number of neurons in LC and corresponding increases of NE cortex fibers or terminals in the forebrain. *In vitro* studies of PC slices have demonstrated that physiological concentrations of exogenous NE dramatically increase excitatory transmission and neural activity in PC; this action is blocked by nonselective a- and ß-blockers (Fain and Garcia-Sainz, 1980; Madison and Nicoll, 1982;1986; Haas and Konnerth, 1983; Collins *et al.*, 1984; Gonzales and Crews, 1985; Gray and Johnston, 1987; Nicoll, 1988). Moreover, NE was also found to significantly increase potassium-evoked release of endogenous EAAs (glutamate, aspartate) from PC slices. These findings support the idea that soman-induced, sustained release of NE in PC could initiate or facilitate seizures by increasing the responsiveness of cortical neurons to EAA and by facilitating EAA release.

3. GLIAL CELLS AND SOMAN NEUROPATHOLOGY

Introduction

It has long been known that many traumatic events culminating in neuropathology are accompanied by dramatic changes in astrocytes. These changes range from the classical observation that glial cells increase in number and density in sites of brain injury (gliosis) to the more recent finding that astrocytes change their morphology by increasing the number and thickness of their processes following injury and deafferentation (Eng and Shiurba, 1987; Reier and Houle, 1988). A watershed discovery was the isolation and characterization of a molecule unique to the cytoskeleton of astrocytes. Antibodies to this molecule (glial fibrillary acidic protein; GFAP) made it possible to selectively label and thus identify astrocytes in histological sections and tissue cultures (Eng and Shiurba, 1987). More important still, was the observation that following injury there was a dramatic increase in the amount of GFAP in astrocytes, doubtless corresponding to an increased production of cytoskeletal protein necessary for the increased number and thickness of astrocyte processes induced by trauma (Amaducci *et al.*, 1981; Eng and Shiurba, 1987; Reier and Houle, 1988). It was shown that GFAP expression was not due to direct injury to the astrocytes. An injury in one part of the brain causing degenerative loss of axons and terminals in a far distant site in the brain caused GFAP expression in astrocytes associated with the degenerating axon terminals. This and many other studies have led to the conclusion that astrocytes "react", i.e., change shape and increase GFAP, in response to signals in neighboring cellular elements (Amaducci *et al.*, 1981; Eng and Shiurba, 1987; Reier and Houle, 1988). The nature of these signals is an area of intense current research.

Methods

Antibodies to three proteins were used: (1) Glial fibrillary acidic protein (GFAP) - GFAP is a cytoskeletal filament specific to astrocytes. Our antibodies (rabbit) to GFAP were supplied by DAKO Corp. (2) OX-41 and OX-42 - These two cell membrane proteins are used to recognize microglia (OX-42) and macrophages (OX-41, OX-42). Our antibodies were supplied by Sera Lab (mouse). Secondary antibodies directed against the species that produced the primary antibody are purchased from several sources according to their affinity and price.

Results

There has been much confusion about the temporal relation between the onset of neuro-traumatic events and the first detectable changes in GFAP. As recently as 3 years ago, it was reported

that 12 hr after the induction of seizures by metrazol, increased GFAP could be detected in the hippocampus (Eriksdotter-Nilsson *et al*, 1987). The significance of this observation was that previous studies had reported increased GFAP increased only after 1-2 days. We decided to examine PC in animals in which soman had caused rapid (30-45 min) induction of *c-fos* in PC neurons. We quickly realized however, that there was a problem with this kind of analysis that was also present in all other previously published studies of the time course of GFAP expression. In normal brain tissue, including PC, there is always some steady-state level of GFAP expression. The presence of GFAP in normal material is an impediment to detecting increased GFAP expression. The problem is a simple signal-to-noise issue; if there is appreciable resident GFAP expression (noise), detection of small, increases (signal) in GFAP is difficult. We solved this problem by performing a series of experiments in which we progressively diluted the concentration of primary antibodies used to detect GFAP to a level (threshold) at which little or no GFAP was detected in sections through PC of normal animals. Using this threshold-dilution method, we analyzed PC sections at various time intervals following soman; we compared animals in which soman had induced *c-fos* in PC neurons with animals that did not convulse and in which *c-fos* expression was low or not present. The results were very striking. In animals with neurons expressing *c-fos* in layer II of PC, astrocytes confined to layer II consistently expressed elevated GFAP as early as 45 min after administration of soman (Figure 1; Shipley *et al*, 1990).

These results warrant two conclusions: (1) Previous studies significantly overestimated the time from the onset of seizures to the first detectable increase in GFAP. The finding that GFAP is increased at 45 min and is coincident with the expression of c-fos in adjacent hyperactive (stressed) neurons indicates that changes in astrocytes that are known to be strongly associated with pathology and cell death are remarkably rapid and parallel the earliest molecular signals of stress in adjacent neurons. (2) The observation that increased GFAP is restricted to layer II in astrocytes forming the immediate microenvironment of the neurons destined to die, taken with the temporally coordinate expression of c-fos in those neurons, leads to the hypothesis that glial changes associated with neuron stress may be causally related to neuron death. The dramatic temporal and spatial coordinate expression of c-fos in PC neurons and GFAP in nearby astrocytes is the earliest known neuropathological event in soman toxicity. By examining animals sacrificed at progressively longer time intervals after soman, we have found that the association of c-fos expression in neurons and increased GFAP in adjacent astrocytes is a correlation that holds invariably across all brain regions examined.

Microglia and Macrophages Macrophages are key cells in tissue pathology; they remove injury-induced cellular debris by phagocytosis. Macrophages are not present in normal brain tissue. It is thought that injury recruits macrophages from circulation and possibly induces the transformation of resident microglia into macrophages (Jordan and Thomas, 1988). A recent study of hippocampal

lesions suggested that an initial event in neuropathology involves microglia secretion of interferon which leads several hours later to the induction of GFAP in reactive astrocytes (Gage *et al.*, 1988; Norgren and Lehmann, 1989). However, our results reported above indicate that GFAP expression is already dramatically increased at 1-2 hr. Thus, we have examined the reactions of microglia following soman using antibodies (OX-41, OX-42; Robinson *et al.*, 1986) that recognize microglia and macrophages.

Our results show that 4-6 hr after seizures, microglia convert to macrophages in PC. This indicates that microglial to macrophage transformation occurs significantly later than the neuronal expression of *c-fos* and astrocyte expression of GFAP/quinolinic acid (QUIN). Taken together, these results suggest that neurons and/or astrocytes may provide the signals for microglial/macrophage reactions to pathology and not vice versa. A critical point, however, is that by 4-6 hr, macrophages are already present in PC. Since macrophages function primarily to phagocytose injury-related cellular debris, it appears, once again, that pathophysiological events are exceedingly rapid in soman-induced seizures. Thus, management of soman-induced seizures, by inactivating the triggers that initiate the seizures, may provide the best protection against brain damage.

Conclusion

Could Astrocytes Contribute to Neuropathology? While early expression of GFAP in astrocytes associated with seizing neurons is indicative of "reactive gliosis," GFAP itself is not likely to be a molecule that figures directly in pathogenesis because GFAP is a structural protein. Increased GFAP expression is, rather, a sensitive signal indicating that the astrocytes are mobilizing other factors which contribute to toxicity. One molecule that may be particularly important in this regard is a tryptophan metabolite, quinolinic acid, which, in the brain, is produced exclusively by astrocytes (Schwarcz *et al.*, 1989). Quinolinic acid (QUIN) first surfaced in relation to the brain when based on its structural properties, it was suggested that QUIN might be a neurotoxin. It is now established that QUIN is a potent neurotoxin (Schwarcz *et al.*, 1989). QUIN is a preferential NMDA receptor agonist (Schwarcz *et al.*, 1989). The synthetic (3-hydroxyanthroanthranilic oxidase; 3-HAO) and degradative (quinolinic acid ribosyltransferase; QRST) enzymes for quinolinic acid are expressed in the brain exclusively by astrocytes (Kohler *et al.*, 1988). Thus, astrocytes both synthesize and degrade a molecule, which is both an NMDA agonist and at slightly higher concentrations, a potent neurotoxin. There is endogenous QUIN in brain homogenates, and the question arises as to the normal function of this neurotoxic molecule and the regulatory controls that normally maintain its expression below toxic levels.

Currently, little is known about the normal function of QUIN. It has been suggested, that since QUIN is an effective agonist for the NMDA receptor, and since NMDA receptor activation has been strongly implicated in prolonging synaptically mediated events, that the local secretion of QUIN by astrocytes, adjacent to active synapses, might provide a "local amplificatory" signal complementing neurally released EAAs to prolong the occupancy or increase the number of occupied NMDA receptors (Schwarcz *et al.*, 1989). Consistent with this, Schwartz (personal communication) has shown that in hippocampal slices incubated in the presence of labelled precursors to QUIN, there is a dramatic increase in labelled QUIN following long-term potentiation (LTP). LTP requires the sustained activation of EAA synapses, and this appears to cause an increase in QUIN synthesis and release. Our hypothesis for the mechanism of soman-induced seizures is that combined cholinergic-noradrenergic hyperstimulation causes cortical neurons to become hyperreactive to EAAs released by synapses on the cortical neurons. By increasing their excitability to EAAs, the neurons discharge more vigorously for the same amount of EAAs released upon them. As a consequence, since these same neurons release EAAs at their own synaptic terminals on other cortical neurons, some of which feed back on the original neurons, cortical circuits are further driven towards an EAA hyperexcitability "chain reactions" to seizures. If QUIN release, as suggested by Schwartz's study, is increased by astrocytes in the vicinity of neurons receiving increased EAA inputs, then the QUIN, by activating NMDA receptors, would further push the EAA chain reaction. Since the neurotoxic potency of QUIN is very great, it is conceivable, therefore, that the sustained hyperactivity produced by soman causes astrocytes associated with seizing neurons to secrete high levels of QUIN. Thus, QUIN would both feed the EAA chain reaction and at the same time have a neurotoxic action. In this event, the release of QUIN under conditions of soman-induced hyperexcitability would function synergistically with EAAs to accelerate the propagation of seizures and cell death.

4. ACTIVATION OF NE NEURONS IN LOCUS COERULEUS BY BOTH SYSTEMIC AND LOCAL APPLICATION OF SOMAN

Introduction

Systemic administration of the irreversible acetylcholinesterase inhibitor soman (pinacolylmethylphosphonofluoridate) produces a rapid (1-2 hr) and profound depletion (70% of control) of olfactory bulb and forebrain norepinephrine (NE) only in animals that undergo convulsions. This depletion is selective for NE, as the levels of other monoamines 1-2 hr after systemic soman injection are not appreciably altered. The pontine nucleus locus coeruleus (LC) is the sole source of noradrenergic innervation of the olfactory bulb and neocortex and is the major source of NE input to the rest of the forebrain. Thus, it is reasonable to hypothesize that the anti-AChE action of soman produces tonic elevation of LC neuronal activity, thus causing depletion of NE in the olfactory bulb and forebrain.

There are several mechanisms by which soman might activate LC neurons. AChE staining, receptor binding, and pharmacologic studies indicate that LC neurons may receive a cholinergic input. Thus, the anti-AChE actions of soman could activate LC neurons by increasing the postsynaptic excitation of a tonically active cholinergic input to LC neurons. Alternatively, soman could activate excitatory afferent inputs to LC. This latter alternative is possible because systemic soman administration produces a variety of peripheral sensory/motor effects that might directly or indirectly influence systems afferent to LC. It is also possible that the brain seizures produced by systemic soman administration might activate LC neurons. Finally, soman may deplete NE by a mode of action that does not involve excitation of LC neurons, for example, by presynaptic effects on LC axonal terminals causing the release of NE.

In order to test these possibilities, the present experiments assessed the effects of systemic and direct application of soman on *c-fos* and AChE staining in LC and on the spontaneous activity of LC neurons *in vivo*. Systemic or intracoerulear application of soman rapidly and potently increased the spontaneous discharge rate of LC neurons; this activation occurred in the absence of seizures. The discharge of LC neurons remained elevated at all post soman intervals examined (up to 2 hr). The maintained excitation caused by soman was completely reversed by systemic administration of the muscarinic receptor antagonist scopolamine, but not by the nicotinic receptor antagonist mecamylamine. Both systemic and intracoerulear administration of soman completely inhibited AChE staining in LC and induced the expression of *c-fos* in LC neurons. These results indicate that LC neuronal activity may be tonically regulated by cholinergic afferent input.

Methods

General Surgical Procedures. Male Sprague-Dawley rats weighing 250 to 375 g were anesthetized with chloral hydrate (400 mg/kg, intraperitoneally [i.p.]). Anesthesia was maintained throughout all procedures with additional injections of 30-40 mg/kg chloral hydrate administered approximately every 30 min. Core body temperature was maintained at 36-38°C with a feedback controlled heating pad.

LC Recordings. Animals were anesthetized with chloral hydrate and placed in a stereotaxic instrument with the skull lowered 12° from the horizontal plane. A hole was drilled in the skull at the coordinates for LC (3.7 mm posterior to lambda, 1.2 mm lateral to midline), and the underlying dura was reflected. A glass recording micropipette (2 to 4-μm tip diameter) filled with 2% pontamine sky blue in 0.5 M sodium acetate was advanced into LC with a hydraulic microdrive. LC neurons were tentatively identified at the time of recording by characteristic impulse waveforms and spontaneous and sensory-evoked discharge patterns, as previously described. Extracellular recordings from individual neurons were amplified and displayed as filtered (300 Hz - 10 KHz bandpass) electrode signals. Impulse activity was monitored with a loudspeaker. Action potentials were isolated from background activity with a waveform discriminator which generated logic pulses for signals that crossed a lower-voltage gate and peaked below an upper-voltage gate. Discriminator logic pulses were led to a computer and chart recorder for on-line data collection. In some experiments, two jewelers screws were threaded into the skull to monitor electroencephalographic (EEG) signals.

Pharmacology. Soman (pinacolylmethylphosphonofluoridate) was injected as a 40-μm solution (73.6 μg/ml, in 0.033% saline; pH 4.5 - 5.0). Microinjections into LC were made with a calibrated glass micropipette (40 to 50-μm tip diameter) that was cemented to a recording micropipette (similar to that described above). The microinjection pipette tip was placed 80 to 200-μm above the tip of the recording electrode. The microinjection pipette was connected to a Picospritzer II (General Valve Corporation) for pressure ejection. Visual inspection of meniscus movement relative to a calibration grid on the injection pipette allowed accurate injection of volumes as small as 15 nl. Typically, boluses of 5-120 nl were injected.

A 26-g needle was inserted into a lateral tail vein for intravenous (i.v.) injection of scopolamine hydrochloride (0.5 mg/ml in distilled water), methylscopolamine bromide (0.5 mg/kg), or mecamylamine hydrochloride (1.0 mg/ml in distilled water). In some experiments, aqueous 5-mg/ml solutions of scopolamine hydrochloride or methylscopolamine bromide were injected intraperitoneally.

c-fos Immunocytochemistry. Animals were deeply anesthetized and perfused transcardially with saline (4°C) for 1 min, followed by 1 liter of 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4, 4°C). Thirty-micrometer-thick sections were cut in the coronal, sagittal or horizontal plane using a vibratome or freezing microtome. Sections were immunocytochemically reacted as follows: (1) sections were rinsed (30 min) in 0.1 M phosphate buffered saline (PBS); (2) placed in 2% normal goat serum (NGS) in PBS containing 0.2 - 2.0% Triton X-100 overnight; (3) incubated in primary antibody (Dr. M. Greenberg, Harvard Univ., rabbit; Cambridge Biochemicals, sheep) at a 1:5,000 dilution in 0.3 Triton X-100 and 2% NGS for 48 hr at 4°C, with constant agitation; (4) rinsed in PBS (30 min) and then incubated in biotinylated IgG directed against the species producing the primary antibody, in PBS (1 hr, room temperature); (5) rinsed in PBS (30 min) and incubated for 1 hr in avidin-biotin-peroxidase complex in 0.1 M PBS and then rinsed in PBS (20 min); and (6) incubated in 0.05% diaminobenzidine (DAB) with 0.1% hydrogen peroxide in PB for 10 min, then rinsed in PB.

Acetylcholinesterase Histochemistry. Immediately after electrophysiological experiments, rats were deeply anesthetized and perfused transcardially with 0.9% sodium chloride containing 0.2% dimethyl sulfoxide until venous return was clear. This was followed by 1 liter of 1% paraformaldehyde, 1.25% glutaraldehyde fixative containing 2% sucrose in 0.1 M phosphate buffer (pH 7.4, room temperature), followed by 1 liter of 10% sucrose in 0.1 M phosphate buffer (pH 7.4, 4° C). Following perfusion, the brain was removed and stored at 4°C in 20% sucrose in 0.1 M phosphate buffer overnight. Serial 40- or 50-μm thick frozen sections were cut into a tray containing 0.1 M phosphate buffer. The sections were processed according to the Koelle-Friedenwald AChE reaction (Koelle and Friedenwald, 1949), as previously described (Van Ooteghem and Shipley, 1984). Prior to histochemical incubation, the sections were washed (7 x 1 min) in distilled water to remove residual phosphates (Van Ooteghem and Shipley, 1984), then incubated with gentle agitation for 2 hr at 37°C in the following solution: 2 mM copper (II) sulfate, 10 mM glycine, 50 mM sodium acetate, 4.2 mM acetylthiocholine iodide, and 0.21 mM ethopropazine. This solution was freshly prepared and carefully brought to final pH of 5.25 with glacial acetic acid. After incubation, sections were again rinsed (7 x 1 min) with distilled water and then reacted (1 min, constant agitation) in a freshly prepared solution of 1% sodium sulfide, carefully adjusted to pH 7.8 with concentrated hydrochloric acid; this reaction was terminated by water rinse (7 x 1 min). The sections were then agitated for 1 min in a freshly prepared solution of 1% silver nitrate, followed by water rinse (3 x 1 min). The sections were stored 3-18 hr in 0.1 M phosphate buffer and rinsed (3 x 1 min) in distilled water prior to mounting from an alcohol-gelatin solution onto subbed slides. Sections were dehydrated through graded alcohols (70%, 95%, 100%, 100%) and xylenes before coverslipping with Permount.

Histology. Micropipette penetrations were marked in most experiments by iontophoretic

ejection of dye with current pulses (-7 uA, 50% duty cycle for 10 min). However, pipette penetrations were not marked in this manner when brains were to be used in *c-fos* experiments; in such cases, electrode tracks and AChE histochemistry were used to localize recording and soman microinjection sites. Animals were then deeply anesthetized and perfused with 10% formaldehyde in 0.1 M phosphate buffer. Brains were removed and stored in a similar solution containing 20% sucrose. Select brain regions were cut into 50 µm-thick frozen sections, mounted on subbed slides, and stained with neutral red. All recording sites were histologically localized from such tissue sections.

Results

Systemic Soman Administration

LC Neuronal Activity. Our recent studies demonstrated that systemic injection of soman in unanesthetized rats produces rapid, profound reduction of forebrain NE in convulsing rats. The soman-induced NE depletion may be due to a tonic excitatory effect of AChE inhibition on LC neurons. To test this possibility, the effect of systemically administered soman on the discharge of LC neurons was examined.

Injection of soman (0.17 mg/kg, im) substantially increased the activity of two LC neurons recorded before and after soman injection; the increase in discharge occurred approximately 7-8 min after injection. However, only 1 or 2 min of postsoman data were collected before the animal died from respiratory failure, probably due to bronchoconstriction and bronchial secretion produced by the anti-AChE action of soman on peripheral autonomic neurons. To circumvent these peripheral effects of soman, rats were pretreated 60-90 min prior to soman injection with the peripherally acting scopolamine analog methylscopolamine bromide (0.5 mg/kg, i.v., n = 2; 1.5 mg/kg, n = 3). Methylscopolamime bromide had no effect on the baseline discharge rate of LC neurons. Since there was no difference between the mean spontaneous discharge rate of LC neurons in intact animals (1.6 ± 0.2 spikes/sec, n = 15) and that in methylscopolamine-pretreated rats (1.6 ± 0.2 spikes/sec, n = 6), data from these two groups were pooled for statistical comparisons. With this peripheral muscarinic blockade, soman elicited a 5- to 10-min period of labored breathing; however, this effect gradually decreased, and LC neurons were recorded as long as 45 min after soman injection.

The spontaneous discharge rates of LC neurons before soman injection ranged from 0.4 to 3.5 spikes/sec, with a mean spontaneous rate of 1.6 ± 0.1 spikes/sec (n = 21, pooled data from above). After soman injection, the range of spontaneous discharge rates for LC neurons was 5.6 to 9.9 spikes/sec. The mean discharge rate for this group, 7.9 ± 0.4 spikes/sec (n = 11), was substantially

greater ($p < 0.01$) than the mean baseline discharge rate of LC neurons.

Soman-induced activation of LC neurons was maintained throughout the period of recordings; there was no difference ($p > 0.9$) between the mean discharge rate of LC neurons examined 8-20 min after soman (7.9 ± 0.5 spikes/sec, n = 5) and that of LC neurons sampled 25-45 min after soman (7.9 ± 0.6 spikes/sec, n = 6). EEG records showed that while soman administration produced a desynchronization of cortical EEG, there was no evidence of seizures (spike activity) at any time during LC recordings.

In one animal tested, administration of the centrally active muscarinic receptor antagonist scopolamine hydrochloride (10 mg/kg; i.p.) rapidly and completely reversed the soman-induced excitation of LC neurons, reducing LC firing rates to control values. There was no difference ($p > 0.4$) between the mean LC discharge prior to soman administration (1.6 ± 0.1 spikes/sec) and that of LC neurons recorded after combined soman and scopolamine hydrochloride administration (1.8 ± 0.3 spikes/sec, n = 9 neurons; Figure 4).

c-fos Staining. Systemic injection of soman (77.6 µg/kg, i.m.) in unanesthetized animals induced rapid and selective expression of *c-fos* in only two areas: LC and PC (Chapter 2). As shown in Figure 3, systemic soman injection caused *c-fos* expression in LC neurons by 30-45 min after injection; this *c-fos* expression was restricted to LC neurons, since c-fos was not expressed in neurons in nuclei surrounding LC, including the medial parabrachial nucleus, mesencephalic trigeminal nucleus, and lateral dorsal tegmental nucleus. With longer post-injection survival times, the number and intensity of *c-fos*-stained LC neurons progressively increased, and *c-fos* expression occurred in other brain regions.

AChE Staining. As shown in Figure 4, AChE staining in LC of normal animals is so intense (Albanese and Butcher, 1979; 1980) that individual neurons cannot be visualized. In contrast, AChE was completely inhibited in LC and other parts of the brain after systemic soman administration.

Intracoerulear Soman Administration

The finding that systemic soman administration increases LC spontaneous discharge rates and induces *c-fos* expression is consistent with the hypothesis that LC neurons may be subject to tonic cholinergic afferent regulation. However, it is also possible that systemically injected soman increases LC activity by nonspecific peripheral sensory/motor effects, or by activation of excitatory afferent inputs

to LC neurons. Therefore, to determine if AChE inhibition focally restricted to LC is sufficient to cause tonic activation of LC neurons, we recorded the spontaneous activity LC neurons before, during, and after microinjection of soman directly into LC.

LC Neuronal Activity. The mean spontaneous discharge rate of LC neurons (n = 22) recorded with a double-barrel recording/microinfusion assembly containing soman (2.2 ± 0.2 spikes/sec) was slightly, but significantly, greater than the mean spontaneous rate of LC neurons recorded with single-barrel pipettes (1.6 ± 0.1 spikes/sec, n = 21). This slightly higher discharge rate was probably due to diffusion of soman from the soman-infusion pipette.

As shown for a typical neuron in Figure 5A, microinjections of small doses (2-6 pmol) of soman into LC resulted in rapid, pronounced, sustained elevation of the spontaneous rate of LC neurons. Increases in LC discharge elicited by one of the smallest doses of soman tested, 15 nl, ranged from 373.3% to 733.3% of control discharge rate, with a mean of 517.7% of control discharge rate (n = 8). Soman-induced elevation of LC discharge was typically observed within 5-20 sec after microinjection and was not associated with any discernible changes in the amplitude or waveform of LC action potentials.

Larger doses of soman elicited more pronounced increases in LC spontaneous rate. For example, the mean discharge rates of LC neurons sampled after cumulative doses of soman ranging from 5 to 120 nl were: 5 nl, 3.8 spikes/sec (n = 2); 15 nl, 5.6 ± 0.9 spikes/sec (n = 8, $p < 0.001$); 30 nl, 5.9 ± 0.9 spikes/sec (n = 5, $p < 0.001$); 60 nl, 7.2 ± 1.1 spikes/sec (n = 4); 90 nl, 9.1 ± 1.7 spikes/sec (n = 4); and 120 nl, 6.6 ± 0.6 spikes/sec (n = 7). Consistent with a recent study by Berridge and Foote (personal communication), soman-induced activation of LC neurons was associated with desynchronization of the cortical EEG. However, there was no evidence of seizure activity in the EEG records at any time during LC recordings after soman administration.

Injection of vehicle alone sometimes elicited a phasic (1-3 min) increase in LC discharge rate, perhaps due to the low ionic concentration or low pH of the vehicle solution. Microinjections of 15 (n = 3) or 30 nl (n = 3) of vehicle, equivalent to volumes of soman that potently and tonically activated LC neurons, elicited only a small, nonsignificant elevation of LC discharge rate (mean discharge rate before and after vehicle injection, 2.1 ± 0.3 and 2.8 ± 0.4 spikes/sec, respectively). In contrast to the tonic excitation of LC neurons produced by soman, the modest increase in LC activity elicited by vehicle microinjection never lasted longer than 2-3 min.

A significant observation with both the systemic and local administration of soman was that the

discharge rate of LC neurons remained elevated as long as examined in these experiments (approximately 2 hr). For example, there was no significant difference between the mean spontaneous discharge rate of LC neurons sampled 5-14 min (7.2 ± 0.6 spikes/sec, n = 16), 15-30 min (5.7 ± 1.0 spikes/sec, n = 5), and 90-120 min after soman (5.4 ± 0.8 spikes/sec, n = 5); data represent firing rates for cells sampled after cumulative doses in excess of 30 nl. Thus, there appeared to be little or no desensitization to or recovery from the excitatory effect of soman within the time periods examined in the present experiments.

Previous studies in anesthetized rats demonstrated that LC neurons exhibit a biphasic response to noxious somatosensory stimuli, such as paw- or tail-pinch (Cederbaum and Aghajanian, 1978; Ennis and Aston-Jones, 1986). This biphasic response consists of a brief burst of 2-5 spikes followed by a postburst inhibition of impulse activity lasting 500-1000 msec (Cederbaum and Aghajanian, 1978; Ennis and Aston-Jones, 1986). Since microinjections of soman markedly increased spontaneous discharge rates, it was of interest to determine if the elevated level of tonic activity altered the responses of LC neurons to such excitatory stimuli. All LC neurons tested exhibited the characteristic biphasic response to a paw- or tail-pinch throughout the prolonged period of soman-induced hyperactivity. Although possible changes in the magnitude of the excitatory or inhibitory components of this response were not quantified, it appeared that a similar burst of tail- or paw-pinch-evoked activity was superimposed upon the soman-evoked increase in spontaneous discharge.

A parsimonious explanation for the pronounced, tonic elevation of LC discharge caused by directly applied soman is that this agent inhibited AChE in LC, causing LC neurons to be exposed to tonically elevated levels of ACh. If this is true, then the cholinergic receptor antagonists should reverse the elevated discharge rates of LC neurons induced by soman. As illustrated in Figure 5C, intravenous injection of the centrally active muscarinic receptor antagonist scopolamine at a dose (0.5 mg/kg, i.v.) previously shown to block excitation of LC neurons to directly applied ACh, rapidly reversed soman-induced elevation of LC discharge. The mean spontaneous discharge rate of LC neurons sampled after doses of 120-240 nl soman followed by scopolamine (2.1 ± 0.4 spikes/sec) did not differ ($p > 0.18$) from the mean baseline discharge rate of LC neurons. In marked contrast, intravenous injection of the nicotinic receptor antagonist mecamylamine (1.0-2.0 mg/kg) did not significantly reduce ($p > 0.8$) the tonic elevation of LC discharge rate induced by 75 nl soman (mean rate after mecamylamine = 6.1 ± 1.1 spikes/sec). It is important to note, however, that previous studies have demonstrated that nicotinic responses in LC are transient and rapidly desensitize (Engberg and Svensson, 1980; Egan and North, 1985). Since the experiments with mecamylamine were conducted at times longer than 3 min after soman microinjection, nicotinic responses subsequent to soman injection may have desensitized by this time.

c-fos Staining. Intracoerulear injection of soman at doses that activated LC neurons (60-120 nl) caused the induction of *c-fos* expression in LC neurons. As shown in Figure 6, LC neurons ipsilateral, but not contralateral, to the injection site had elevated *c-fos* expression. In contrast, there was little if any *c-fos* expression in neurons in nuclei adjacent to LC (i.e., medial parabrachial nucleus, mesencephalic trigeminal nucleus, and lateral dorsal tegmental nucleus).

AChE Staining. As shown in Figure 2, acetylcholinesterase histochemistry demonstrated that microinjections of soman into LC nearly completely inhibited AChE staining in LC and the adjacent pericoerulear area (n = 4). With small microinjections of soman (less than cumulative doses of 90 nl), AChE inhibition was limited to LC proper, the rostromedial pericoerulear region, including the lateral dorsal tegmental nucleus and Barrington's nucleus, the mesencephalic trigeminal nucleus, and the medial border of the parabrachial complex. With larger microinjections of soman (120-240 nl), inhibition of AChE staining extended further laterally into the parabrachial complex, rostromedially into the dorsal tegmental nucleus, and caudally into the medial vestibular nucleus.

Discussion

The present findings demonstrate that systemic or focal intracoerulear microinjection of small doses of the irreversible acetylcholinesterase inhibitor soman potently increases the discharge rate of LC neurons. Following either mode of administration, LC spontaneous rates remained elevated at the longest postsoman time interval examined (up to 2 hr), consistent with the irreversible actions of the AChE inhibitor. The maintained activation of LC neurons evoked by soman was rapidly and completely reversed by the muscarinic receptor antagonist scopolamine, but not by the nicotinic receptor antagonist mecamylamine. A molecular correlate of this tonically elevated physiologic activity was the induction of *c-fos* expression in LC neurons. These overall results indicate that LC neurons *in vivo* may receive tonic cholinergic input.

Various lines of evidence, although largely indirect, suggest that LC neurons receive a cholinergic input. LC neurons stain intensely for the degradative enzyme AChE. Indeed, the AChE staining in LC is one of the most heavy in the brain (Albanese and Butcher, 1980). Receptor binding studies show that LC contains both muscarinic and nicotinic receptor subtypes (Rotter *et al.*, 1979). Biochemical measurements indicate that the biosynthetic enzyme for ACh, choline acetyltransferase (ChAT), is present, presumably, in fibers in the LC area. However, such measurements may have been contaminated by ChAT contained in cholinergic neurons located adjacent to LC in the lateral dorsal tegmental nucleus. It is interesting that a recent study of immunocytochemical localization of ChAT-stained fibers indicated that the LC nucleus proper is nearly devoid of ChAT-positive fibers; however, the

pericoerulear region rostromedially adjacent to LC contains a considerable plexus of ChAT-positive fibers. We have recently demonstrated that this same rostromedial pericoerulear region contains a dense plexus of LC extranuclear neuronal dendrites (see below).

Previous *in vivo* pharmacologic studies indicate that LC neurons are potently excited by direct application of acetylcholine and muscarinic analogs (Guyenet and Aghajanian, 1979; Engberg and Svennson, 1980; Adams and Foote, 1988). Such excitation was completely blocked by direct application of the muscarinic receptor antagonist scopolamine. Engberg and Svennson (1980) reported that systemic, but not iontophoretic, administration of nicotine excited LC neurons. However, experiments *in vitro* demonstrate that ACh excites LC neurons through both nicotinic and muscarinic receptors (Engberg and Svensson, 1980; Egan and North, 1985, 1986). In the presence of muscarinic antagonists, nicotine produces a fast, brief depolarization of LC neurons that exhibits rapid desensitization (Egan and North, 1986). Nicotine-evoked responses were blocked by hexamethonium, but not by a-bungarotoxin (ref). In contrast, muscarinic responses have a longer time course and do not appear to desensitize (ref). Thus, pharmacologic experiments indicate that ACh can excite LC neurons via activation of muscarinic or nicotinic receptors.

In the present experiments, LC neurons were robustly activated after peripheral injection of the irreversible AChE inhibitor soman. While soman produces a host of peripheral cholinomimetic side effects (e.g., labored respiration, hypoxia, gastrointestinal stimulation, hypotension, bradycardia, etc.) that may affect LC activity, soman activated LC neurons despite pretreatment with the peripherally acting muscarinic receptor antagonist methylscopolamine-bromide. The increase in spontaneous discharge rate of LC neurons produced by soman in methylscopolamine bromide-pretreated animals was identical to that in animals that did not receive this pretreatment. In addition, the range of LC discharge rates following systemic soman administration was essentially the same as that following intracoerulear microinjection of soman. Thus, increased LC firing rates produced by systemically administered soman appear to be mediated by central cholinergic stimulation of LC neurons. This possibility is further supported by the observation that the soman-induced, tonic activation of LC neurons was completely reversed by the centrally active muscarinic receptor antagonist scopolamine hydrochloride.

In support of the hypothesis that activation of LC neurons following systemically administered soman is mediated by facilitation of direct cholinergic stimulation within LC, we found that focal infusion of picomolar concentrations of soman directly into LC robustly activated every LC neuron tested. The finding that LC neurons were activated after injections of soman as small as 5-15 nl indicates that this activation was primarily mediated by facilitation of cholinergic transmission at synapses on the cell bodies or proximal dendrites of LC neurons. This possibility is supported by the

histochemical finding that intracoerulear microinjection of volumes of soman as large 90 nl produced a focal zone of AChE inhibition restricted almost entirely to the LC nucleus. It is likely, however, that the progressive increases in LC firing rates with incremental doses of soman may have been due to additional facilitation of transmission at cholinergic synapses on the more distal dendrites of LC neurons, including those that extend beyond the boundaries of LC proper (see below). However, despite the diffusion of soman into adjacent structures with larger injections, *c-fos* staining revealed that LC neurons were the only cells in the dorsolateral pons to exhibit elevated expression of this transcriptional regulatory protein.

Similar to results with systemic injections, intracoerulear infusion of soman tonically activated LC neurons. LC discharge rates were significantly elevated at all times sampled in these experiments (up to 2 hr), with only a small decrease in firing rate after the first 15 min of soman injection. This maintained activation of LC neurons was completely reversed by intravenous injection of scopolamine hydrochloride at a dose previously shown to antagonize LC responses to directly applied ACh. This result indicates that the maintained activation of LC neurons by soman is mediated by muscarinic receptors. In agreement with this possibility, tonic activation was not significantly affected after injection of the nicotinic receptor antagonist mecamylamine. However, as noted above, studies *in vitro* indicate that nicotinic excitation of LC neurons undergoes rapid desensitization. Thus, it is possible that the initial phase of soman-induced activation of LC has a nicotinic component, although the present studies did not address this possibility.

The most parsimonious explanation for increased LC firing rates after soman administration is that soman's inhibition of AChE facilitates the excitation of LC neurons to a tonically active cholinergic input. However, there are several alternative possibilities. (1) Soman could activate excitatory afferent inputs to LC. For example, AChE inhibition could lead to accumulated ACh which, in turn, could cause the release of excitatory amino acids from the terminals of excitatory amino acid inputs to LC. (2) Alternatively, AChE inhibition could inhibit intracoerulear NE release from LC axonal and dendritic terminals or inhibit second messengers of the intracoerulear a-2-autoreceptor, thereby decreasing inhibitory collateral interactions among LC neurons (disinhibition). Future experiments will address these possibilities. (3) Soman could activate local neurons in the pericoerulear area that, in turn, have excitatory connections with LC neurons. This possibility seems unlikely because increases in LC discharge were obtained with injections of soman as small a 5 nl. In addition, AChE staining revealed that doses of soman as large as 90 nl produce a focal zone of AChE inhibition largely restricted to LC, and *c-fos* immunocytochemistry demonstrated that LC neurons were the only neurons to exhibit elevated expression of *c-fos* even after large microinjections of soman that spread beyond the borders of LC.

The present observation that direct injection of an AChE inhibitor potently increases the spontaneous discharge of LC neurons thus suggests that LC neurons receive a tonically active cholinergic input. If this is true, then direct or systemic administration of the muscarinic antagonist scopolamine might be expected to increase the spontaneous rate of LC neurons, but this has not been observed (Engberg and Svennson, 1980; Ennis and Aston-Jones, 1986), except with near-lethal doses of this agent. The reason for this discrepancy is unclear, but it is possible that high levels of AChE expressed by LC neurons normally function to rapidly degrade ACh tonically released by cholinergic afferent synapses, minimizing its postsynaptic activation of LC neurons.

The source(s) of possible cholinergic input to LC neurons have not been directly identified with double-labelling techniques. ChAT-positive cell bodies are located in the medulla in nucleus prepositus hypoglossi and nucleus paragigantocellularis, the two major afferent inputs to LC (Aston-Jones *et al.*, 1986). Both of these medullary regions project heavily to the central core of LC proper as well as to the pontine tegmentum rostral and medial (rostromedial pericoerulear zone) to LC proper (Aston-Jones *et al.*, 1986). As noted earlier, a recent comprehensive study of cholinergic terminals and cell bodies demonstrated that LC proper contains only sparse ChAT-positive fibers and terminals. In contrast, the rostromedial pericoerulear adjacent to LC contains a fairly dense plexus of ChAT-positive fibers, indicating that this region may receive strong cholinergic innervation. In addition, there are local ChAT-positive neurons in this same region in the lateral dorsal tegmental nucleus. It is noteworthy that the rostromedial pericoerulear region contains a rich plexus of extranuclear LC dendrites. Thus, there is extensive spatial overlap between the distal, extranuclear dendrites of LC neurons and ChAT-positive fibers in the rostromedial pericoerulear region. Anatomical studies are under way to determine which of these possible systems provide cholinergic inputs to LC.

Recent experiments in this laboratory have demonstrated that systemic injections of soman that induce convulsions reduced forebrain levels of NE levels by as much as 50% from control values within 50 min after peripheral soman administration; NE levels were not altered in animals receiving the same dose of soman but not exhibiting convulsive activity. Within 2 hr of soman administration, forebrain NE in convulsing animals was reduced by greater than 70% in convulsing animals (El-Etri, *et al.*, 1990; Shipley *et al.*, 1990; Chapter 5). The reduced forebrain NE after systemic soman administration may have been due to a tonic cholinergic stimulation of LC neurons leading to depletion of NE in LC terminals. However, as this depletion was obtained only in convulsing animals, it was possible that generalized brain seizures, and not cholinergic stimulation, produce hyperstimulation of LC neurons. However, the present studies demonstrated that both systemic or intracoerulear microinjection of soman can robustly and tonically activate LC in the absence of seizures. Experiments are in progress to assess the effects of direct intracoerulear injection of soman on forebrain NE levels.

In summary, the present study demonstrates that direct application of an AChE inhibitor potently activates LC neurons. Thus, cholinergic input to LC may be important in regulating the activity of LC neurons. Additional experiments to determine the source and terminal pattern of possible cholinergic inputs to LC are in progress.

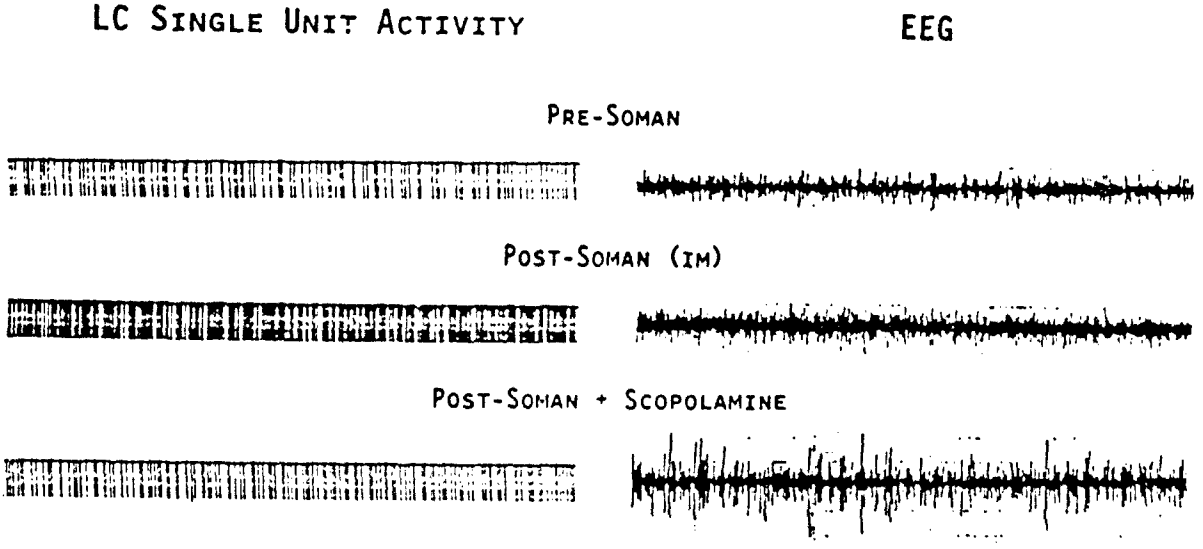


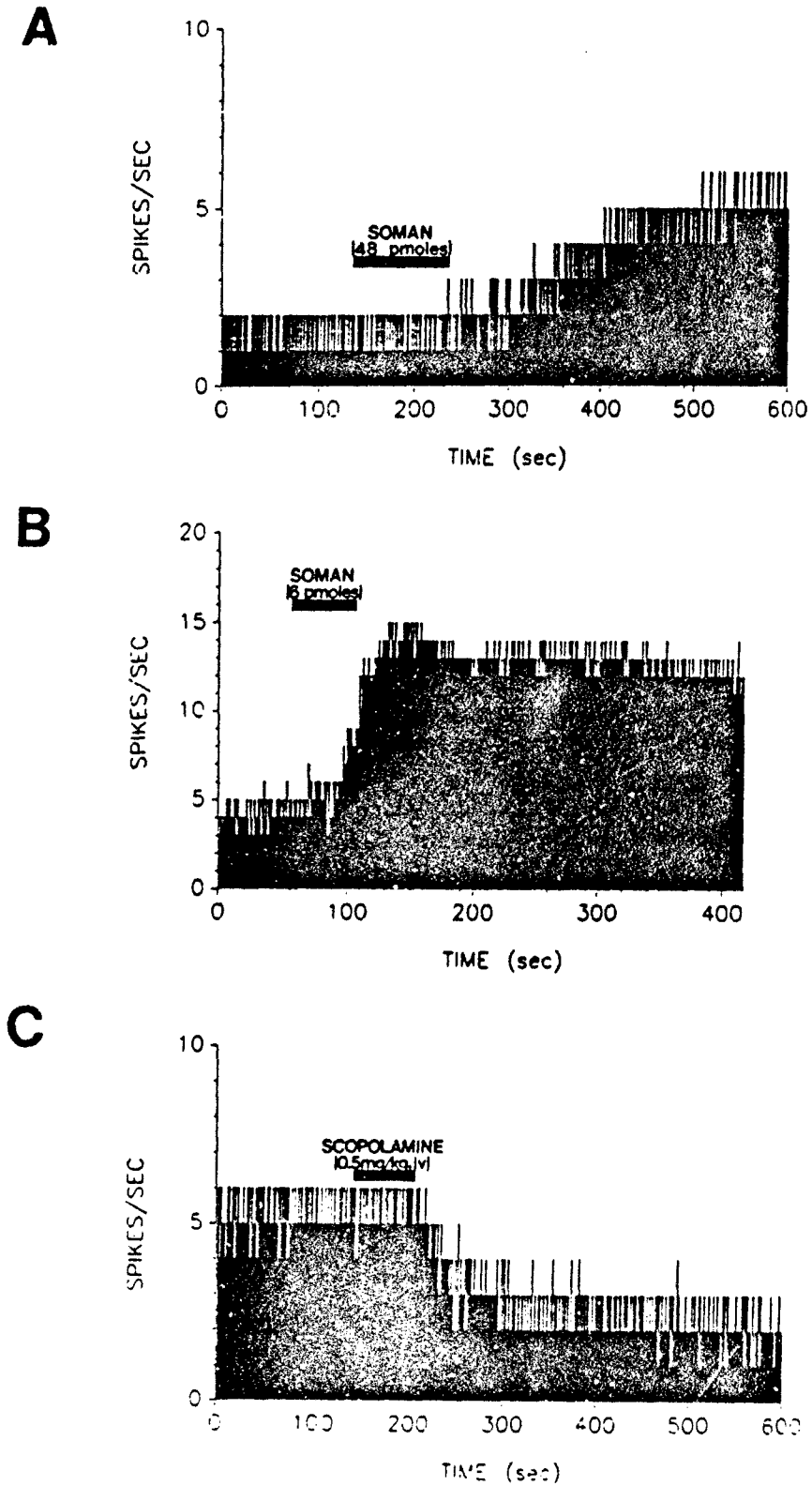
Figure 4. Effect of Systemic Injection of Soman and Scopolamine on Locus Coeruleus (LC) Neuron Firing Rate and Electroencephalogram (EEG).

Figure 5. Microinjection of Soman Directly into Locus Coeruleus (LC) Tonically Activates LC Neurons via Muscarinic Cholinergic Receptors.

A: Computer ratemeter records showing the spontaneous activity of a single locus coeruleus (LC) neuron before and after microinjection of a single dose of soman into LC. Before application of soman, this typical LC neuron has a slow and regular spontaneous discharge rate of 1.5 spikes/sec. Microinjection of 48 pmol of soman (in 120 nl, shown at upper bar) from a micropipette positioned 150 mm above the tip of the recording microelectrode (see text for details) rapidly increased the spontaneous discharge of this neurons to approximately 5.5 spikes/sec; the spontaneous rate of this neuron continued to increase to a final rate of 7 spikes/sec, not shown).

B: Computer ratemeter records showing the spontaneous discharge of a LC neuron; recordings from this LC neuron were initiated 7 min after microinjection of soman (12 pmol) while recording another LC neuron. The spontaneous rate of this neuron after this first dose of soman was 4.0 spikes/sec. Microinjection of a second, very small dose of soman (6 pmol in 15 nl, shown at upper bar) from an adjacent micropipette positioned 100 µm above the recording electrode increased the spontaneous rate of this cell to 12.9 spikes/sec. Overall, intracoerulear microinjection of soman (2-96 pmol) significantly ($p < 0.0001$) increased the spontaneous rate of LC neurons ($n = 64$). As shown in A and B, microinjection of soman into LC tonically activated LC neurons; the discharge rates of all LC neurons examined was significantly elevated at all time intervals examined in the 2-hr postsoman epoch studied in these experiments.

C: Computer ratemeter records showing tonic elevation of spontaneous discharge of an LC neuron 30 min after microinjection of soman (96 pmol) into LC. Intravenous administration of the muscarinic cholinergic antagonist scopolamine (0.5 mg/kg) rapidly reversed the soman-induced tonic elevation of the discharge rate of this LC neuron from 5.5 spikes/sec to 2.0 spikes/sec; the mean control discharge rate of LC neurons is 1.9 spikes/sec. Overall, scopolamine (0.5mg/kg, i.v.) completely reversed soman-evoked activation of LC discharge ($n = 8$, $p < 0.001$).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(4.8 pmoles)
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(0.5mg/kg, IV)
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500
600
TIME (sec)

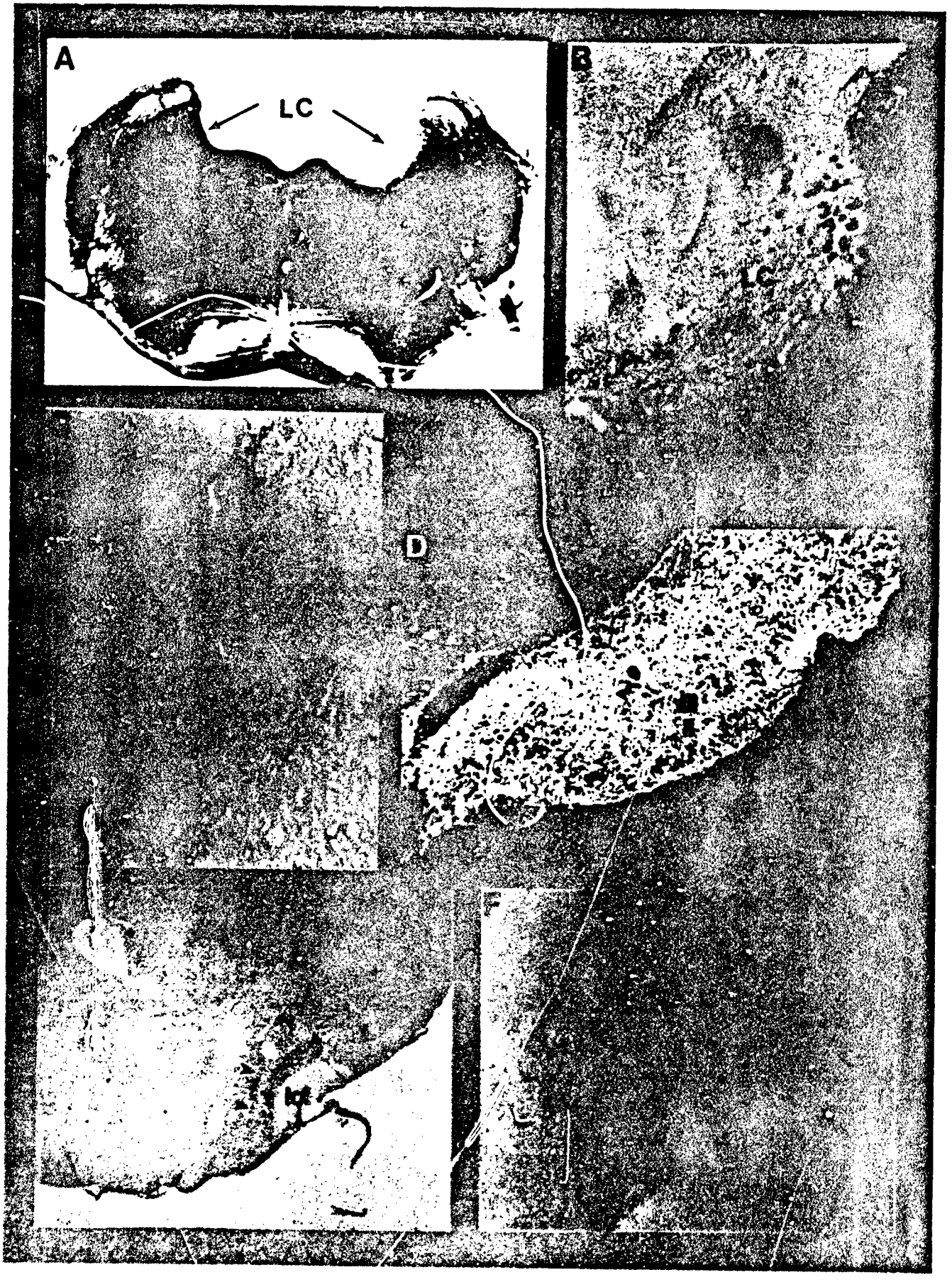
Figure 6. Microinjection of Soman into Locus Coeruleus (LC): (1) Inhibits Acetylcholinesterase (AChE) Staining, (2) Causes Intense Sustained Activation of LC Neurons, and (3) Induces *c-Fos* Expression in LC and Piriform Cortex (PC).

A and B: AChE stained section through LC from an experiment in which soman was microinjected into LC. AChE staining in the left, intact side (at arrow) is normal. On the right side (at arrow), shown at higher magnification in panel B, AChE is completely inhibited in most LC neurons. This section is taken from the animal in which LC neuronal activity was recorded before and after the microinjection of soman (see Figure 5).

C: Microinjection of soman into LC induces *c-fos* expression in LC. Two hours after the microinjection of soman, during which single LC neurons were continuously recorded, the animal was sacrificed and adjacent section were stained for AChE (panels A and B) and *c-fos* (panel C). Note that 2 hr of local, restricted AChE inhibition induced *c-fos* expression in the majority of LC neurons, but not in adjacent neurons.

D: Piriform cortex (PC) is heavily innervated by noradrenergic fibers (stained by DBH immunohistochemistry). These fibers all arise from LC; noradrenergic fibers are somewhat more dense in layers II and III than in other layers of PC.

E and F: Intracoerulear microinjection of soman activates LC neurons (see Figure 5) and induces the expression of *c-fos* in layer II in PC. These sections through PC are from the same case depicted in panels A-C. Two hours of sustained activation of LC neurons induces *c-fos* expression preferentially in layer II of PC (shown at arrowheads). (LOT, lateral olfactory tract)


A
LC
B
C
D

5. RAPID DEPRESSION OF NE LEVELS AFTER SOMAN INTOXICATION IN CONVULSING BUT NOT NONCONVULSING RATS

Introduction

At sufficient doses, organophosphate (OP) toxins, including the chemical nerve agent soman cause convulsions, neuropathology, and, ultimately, death (Jovic, 1973; Lemerceier *et al.*, 1983; McLeod *et al.*, 1984). A major problem in treating OP intoxication is that peripherally-acting pharmacological agents which prevent death do not prevent seizures. Soman-induced seizures are especially prominent in cortical structures and can lead to irreversible brain damage. Although a primary cause of these symptoms is the excess of ACh which follows AChE inhibition, centrally acting muscarinic blockers, such as atropine, alleviate, but do not block, the convulsive actions of OP nerve agents (McDonough *et al.*, 1989). There is a relatively weak relationship between reductions of AChE and the incidence of convulsions (Hoskins *et al.*, 1986; present study). Likewise, there is little systematic relation between ACh and GABA levels and soman-induced convulsions (Liu *et al.*, 1988). These results suggest that OP-induced convulsions may not be due solely to changes in ACh or GABA.

There are reasons to suspect that the neuromodulatory transmitter norepinephrine (NE) may be involved in the generation or maintenance of OP-induced convulsions. Noradrenergic neurons in the locus coeruleus (LC) send fibers to all parts of the forebrain and are the sole source of NE in the forebrain (Foote *et al.*, 1983); thus, LC is able to exert a global influence on neuronal excitability. LC neurons contain high levels of AChE (Albanese and Butcher, 1979) and cholinergic agonists strongly increase the firing rates of LC neurons (Engberg and Svensson, 1980; Egan and North, 1985, 1986; Adams and Foote, 1988). Recently, we have shown that microinjection of soman into LC increases the mean firing rate of LC neurons to five times the control rate. This increased firing rate is sustained for several hours (El-Etri *et al.*, 1990; Ennis and Shipley, in preparation). Systemic injection of soman causes a comparable sustained increase in the firing rate of LC neurons (El-Etri *et al.*, 1990; Ennis and Shipley, in preparation).

Taken together, these findings suggest that there could be rapid, sustained release of NE in soman intoxicated animals as the result of increased firing of LC neurons. Other results suggest that NE release may have important effects on the generation of OP-induced seizures. Brain NE declines rapidly in guinea pigs receiving a seizurogenic dose of soman (Fosbraey *et al.*, 1990) and in rabbits intoxicated with another irreversible AChE inhibitor diisopropylfluorophosphate (DFP) (Glisson *et al.*, 1974). In contrast, other monoamine transmitters are relatively little affected. The α_2-noradrenergic agonist, clonidine, protects as well as atropine against soman-induced convulsions (but not death) and is

synergistic with atropine in preventing seizures (Buccafusco, 1984; Aronstam *et al.*, 1986; Buccafusco and Aronstam, 1986; Buccafusco *et al.*, 1988). Clonidine reduces release of ACh from cholinergic terminals (Vizi, 1980). However, clonidine also inhibits firing of LC neurons (Adams and Foote, 1988; Marwaha and Aghajanian, 1982). Thus, the protective action of clonidine could be due to its direct action on LC neurons.

In view of the relatively loose relation between ACh or GABA levels and convulsions and the evidence suggesting a role of NE in OP-induced seizures, it was of interest to determine the relationship between the degree of NE depletion and the occurrence of convulsions following soman intoxication. To do this, we determined the effects of a single dose (78 μg/kg) of soman on forebrain NE levels at six time intervals in the 4 days following intoxication. At this dose, 68% of the animals developed convulsive seizures. Presence or absence of seizures was noted and later correlated with NE levels. Two separate regions of the brain from each rat were analyzed in order to provide an internal control for experimental variation. The two regions chosen were the olfactory bulb, which receives a strong noradrenergic projection from LC (Fallon and Moore, 1978; Halasz *et al.*, 1978; Shipley *et al.*, 1985; McLean *et al.*, 1989), and the rostral forebrain, including the PC which is the most vulnerable site for soman-induced seizures and neuropathology.

Methods

Chemicals. Soman (pinacolylmethylphosphonofluoridate) was supplied by the U.S. Army Institute for Chemical Defense, Aberdeen Proving Ground, Maryland. Monochloroacetic acid, octyl sodium sulfate, all the biogenic amines, including norepinephrine bitartrate, and the internal standard 3,4-dihydroxybenzylamine hydrobromide (DHBA) were obtained from Sigma Chemical Co. Fisher Scientific was the source of L-cysteine, Na_2EDTA, perchloric acid, and high performance liquid chromatography (HPLC) grade acetonitrile.

Soman Administration. Adult male Sprague-Dawley rats were obtained from Harlan Laboratories, Indianapolis, IN. After arrival, the animals were housed in pairs under controlled conditions with free access to food and water at least 1 wk before use. Rats weighed 290-340 gm on arrival and reached 300-400 gm by the time of injection. A single 78 μg/kg dose of soman in saline was injected i.m. into the left rear leg. This dose is approximately 65% of the dose reported necessary to cause convulsions in 50% of injected rats (ED_{50}; Liu et al. 1988; Wade *et al.*, 1988). For each survival period a control group of rats was injected with a proportional volume of vehicle.

Scoring of Convulsive versus Nonconvulsive Rats. Soman injected rats were observed every 5 min in the first 30 min after injection and at longer intervals during the designated survival period. For each injected rat, the presence or absence of convulsions was noted during this observation period. Injected animals fell into clearly distinguishable convulsive and non-convulsive groups. Those animals which developed convulsions did so within 30 min and continued to be convulsive for the next several hours (see Results). This record was used for later data analysis.

Animal Procedure and Tissue Dissection. Rats were sacrificed by decapitation at 1, 2, 4, 8, 24, and 96 hr following soman administration. The brains were rapidly removed and the olfactory bulbs were separated from the remainder of the brain by cutting the olfactory peduncle. The cerebellum and underlying brainstem were removed with a vertical knife cut and the forebrain was divided at the midline. The remaining brainstem including the thalamus and hypothalamus was then removed by pinching with a pair of curved forceps. This sample contained a small remnant of the basal ganglia. Forebrain samples (400-600 mg) and two olfactory bulbs (50-80 mg) were placed in plastic tubes, immediately frozen on dry ice, and stored at -80°C until assay.

Forebrain samples were homogenized using an ultrasonic tissue disrupter (Branson Sonic Power, Danbury, CT) at 0°C for 2 min. in 1.C ml of 0.10 M perchloric acid containing 0.1% cysteine as anti-oxidant and 10 ng/ml of the internal standard (DHBA). About 200 µl of individual homogenates were transferred to an Eppendorf polypropylene tube and centrifuged at 1360 g for 7 min at 4°C. One hundred µl of the supernatant was diluted with 200 µl of the homogenization solvent. The olfactory bulbs were treated identically except that the bulbs were homogenized for 1 min in 200 µl of solvent. For either tissue, 15 µl of the final solution was injected into the HPLC.

Chromatographic Methods and Assay of Norepinephrine. NE concentrations were determined using a Bioanalytical Systems (West Lafayette, IN) Model 200 Liquid Chromatograph, equipped with a a Model LC-4B electrochemical detector (Bioanalytical Systems). The glassy carbon working electrode was kept at 0.67 V against an Ag/AgCl reference electrode. Full-scale sensitivity was 10 nA. Chromatographic separations were effected with a 100 x 3.2 mm Biophase ODS 3-µm (C_{18}) column (Bioanalytical Systems). The mobile phase consisted of 0.06 M monochloroacetic acid (pH 3.0), 1.20 mM octyl sodium sulfate (SOS) and 0.1 mM Na_2EDTA in the aqueous phase and 1.5% acetonitrile in the organic phase. The column and the electrochemical cell were held at 40 and 41°C, respectively. The mobile phase flow rate was 0.9 ml/min. The instrument was calibrated daily using five 15 µl standards containing 0.3-1.5 ng NE and 0.3 ng of the internal standard DHBA.

Determination of AChE. In a second experiment, the relation between residual AChE and NE

depletion was measured. Rats were injected with the same dose of soman used in the first experiment. A set of sham-injected animals (n=11) was processed in an identical manner. The animals were sacrificed by decapitation 1 hr after injection of soman or vehicle. The forebrains were isolated as described previously, and were divided sagitally at the midline. One hemisphere was processed for monoamines as described previously. The second hemisphere was analyzed for AChE activity. To determine AChE activity the brains were homogenized in a Polytron homogenizer containing the following buffer (10 ml/gm tissue): NaCl, 1.0 M; EDTA, 0.2 mM; Tris HCl (pH 7.4), 50.0 mM; Triton X-100, 1%. The homogenates were centrifuged at 100,000 g for 30 min and the supernatants were removed for acetylcholinesterase (AChE) assay (in duplicate) using the Ellman procedure (Ellman *et al.*, 1961) with 0.45 mM acetylthiocholine. All assay tubes contained 50 µM iso-OMPA to inhibit pseudocholinesterase.

Results

Incidence and Signs of Soman-induced Convulsions. All soman-injected animals exhibited signs of intoxication. Nonconvulsive animals were inactive and unresponsive to stimuli. Convulsive animals exhibited tonic shaking movements of the head and body, sometimes associated with excess salivation and labored breathing.

Convulsions were observed in 68% of injected animals. This incidence of convulsions is similar to that obtained by Wade et al (1987) using a similar dose of soman (58% convulsive with a dose of 81 µg/kg). These signs first appeared 15-20 min after injection and gradually increased in intensity. Convulsive activity was intense between 1 and 4 hr and declined thereafter. All animals which developed convulsions during the post-soman survival period had done so by 30 min following injection; no additional rats developed convulsions after this period. In the 8-hr survival group, two of seven convulsive rats recovered from convulsions; convulsive activity in the remaining 5 rats was reduced in intensity at the time of sacrifice. In the 24 and 96 hr groups, all animals had recovered from convulsions. In the 96 hr group, surviving convulsive rats had lost 30% of body weight; non-convulsive rats in this group maintained their body weight. Mortality was insignificant in the 1 to 8 hr groups (3 of 71); however, there was significant mortality in the 24 hr (20 of 36 animals; 55%) and 96 hr (24 of 36 animals; 67%) groups. This mortality is comparable to the 46% observed by Shih (1982) following a somewhat larger (120 µg/kg) dose of soman. All the animals which died prior to sacrifice had exhibited convulsions.

Relation between NE Levels and Behavioral Convulsions. NE levels in olfactory bulb and forebrain of all convulsive animals were profoundly depressed (Figure 7, Tables 1 and 2). At 1 hr after

soman injection, forebrain NE was 50% of control. The reduction in NE was maximal at 2 hr, when forebrain NE was only one third (32%) of the control level. Maximum depression of NE in the olfactory bulb of convulsing animals was slightly less than in forebrain, but still large (43% of control). NE levels remained severely depressed at 4 hr after intoxication. NE recovered over the next 4 days; at 96 hr after intoxication, NE levels were very near control levels. The increase in NE levels between 4 and 8 hr was statistically significant (Student's t-test, $p<0.0001$; Sheffe's F-test, $p<0.01$), indicating that recovery of NE began between 4 and 8 hr after intoxication.

In contrast, NE levels in non-convulsing rats remained very near control levels at all time points examined. The largest decrease from control levels was in the 1 hr survival group; in this group, mean forebrain NE was 87% of control. This difference between the experimental and control groups was weakly significant ($p<0.05$), but was not significant when tested with a method which corrects for multiple comparisons (Sheffe's F-test, $p>0.05$). In the olfactory bulb samples from this group, the mean NE was less than one SEM from control and was therefore not significant. A depression of NE levels in non-convulsive animals at 1 hr after intoxication is, therefore possible, but not certain from the present data. NE levels in non-convulsive animals at later time points were not significantly different from control.

The strength of the correlation between convulsive activity and NE levels is further illustrated in Figure 8, in which the bulb and forebrain NE concentrations for individual animals in the 2 and 4 hr survival groups are plotted. Convulsive and nonconvulsive animals clearly fall into two distinct, non-overlapping, groups. In all convulsive animals NE levels were severely reduced, while in all non-convulsive animals forebrain NE levels were nearly normal. There is a strong correlation ($R^2 = 0.83$) between NE levels in bulb and forebrain of individual animals.

Correlation of AChE Inhibition, NE Depletion and Convulsions. A possible explanation for the observation that the degree of NE depletion falls into distinct groups is that the degree of brain AChE inhibition after soman intoxication also falls into distinct groups. This might occur, for example, as the result of variability in the location of the injected drug relative to major vessels in the injected muscle. Alternatively, inhibition of AChE might be approximately constant in all injected animals, but the development of convulsions might depend on other factors. To assess these possibilities, we conducted a second study in which we measured both forebrain catecholamine content and residual AChE activity in a population of rats injected intramuscularly with soman. A single survival time of 1 hr was used.

The relation between residual AChE activity, NE and convulsions is presented in Figure 9, in which measured NE and AChE levels for individual brains are normalized against the mean of a control

group processed simultaneously. Mean values for these groups are presented in Table 3. As in the first experiment, NE levels fell into two clearly defined groups: In animals scored as convulsive, NE was always less than 50% of control (mean 38% of control), but in nonconvulsive animals, NE levels were near normal (mean 100% of control). There was wide variation in residual AChE; however, unlike NE levels, there is overlap between the AChE levels of convulsive and nonconvulsive animals. Thus, the NE level is a better predictor of the occurrence of soman induced convulsions than AChE levels. The threshold for seizures is about 30% residual AChE activity.

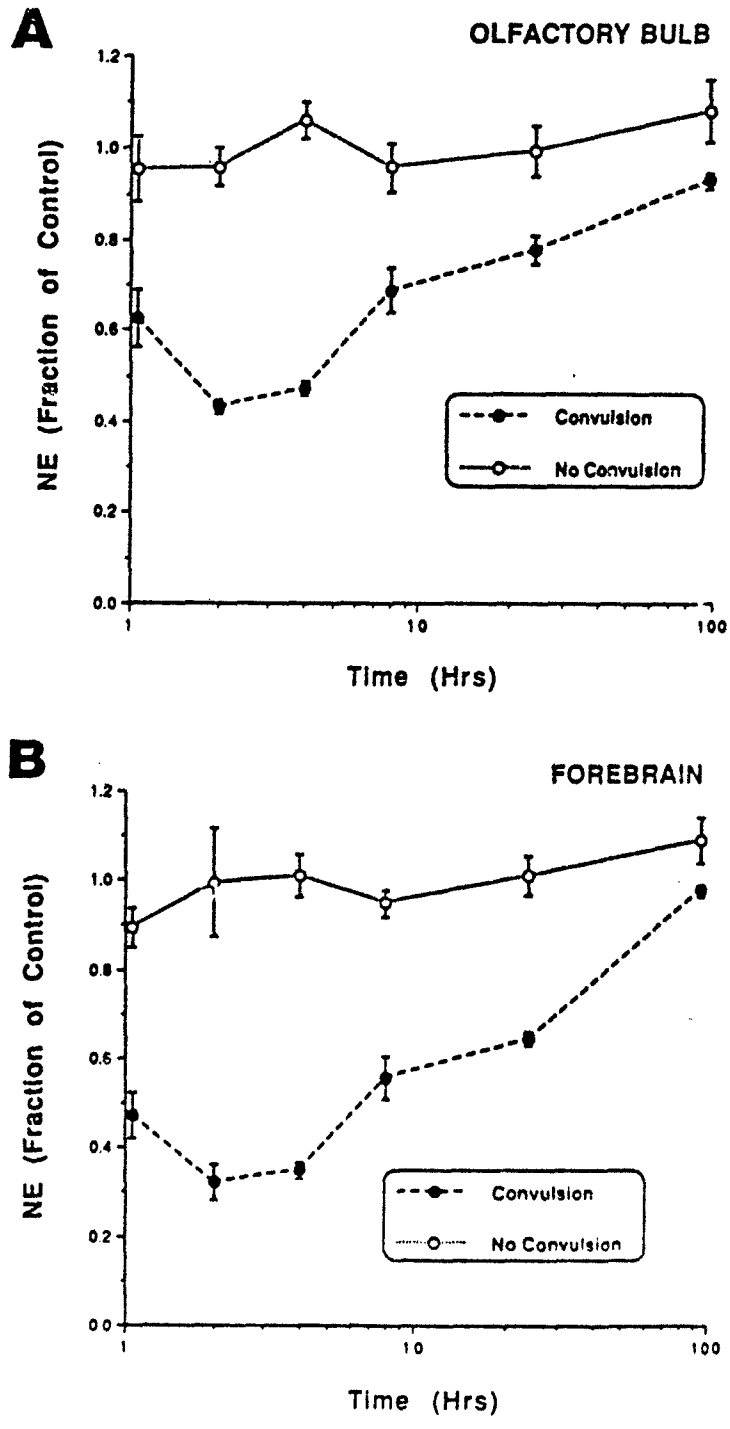


Figure 7. Differences in Norepinephrine (NE) Levels in Convulsing and Nonconvulsing Animals after a Single 78 μg/kg soman injection. A) olfactory bulb. B) forebrain. Time axis is logarithmic.

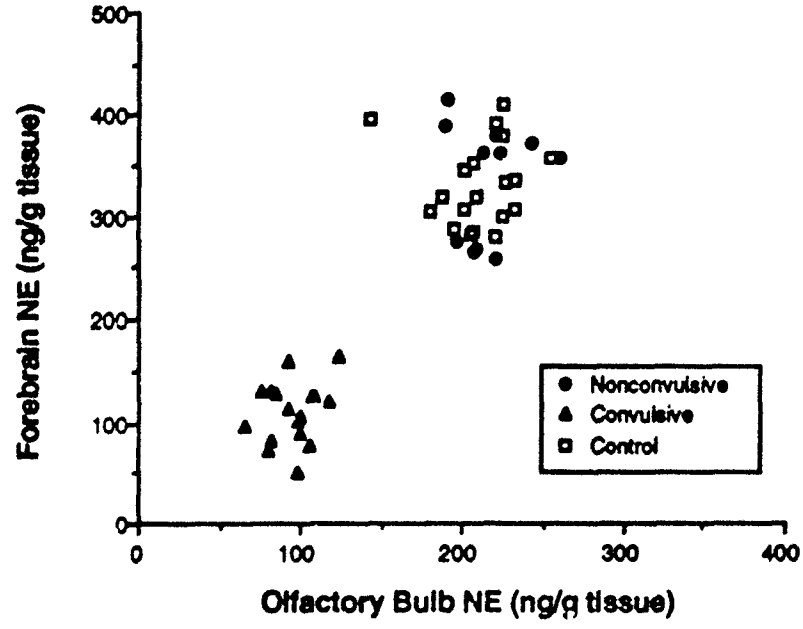


Figure 8. Scatter Plot of Norepinephrine (NE) Levels (ng/g tissue) in Olfactory Bulb and Forebrain of Convulsive and Nonconvulsive rats after a single 78 µg/kg Soman Injection. Olfactory bulb vs forebrain NE levels for all individual rats in the 2- and 4-hr survival groups are plotted. Convulsive and nonconvulsive animals fall into nonoverlapping groups. (R^2=0.83).

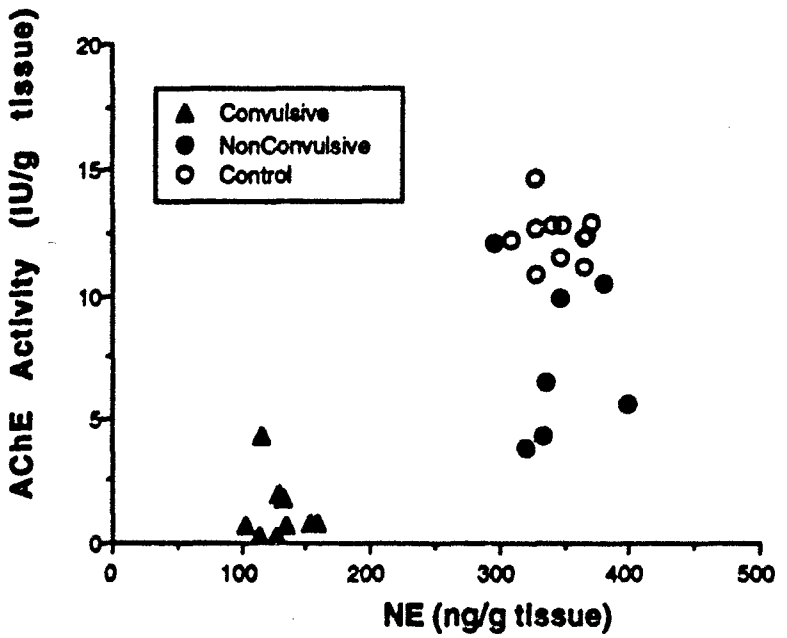


Figure 9. Correlation between Acetylcholine (AChE) Inhibition, Norepinephrine (NE) Depletion, and Convulsions.

Table 1

Forebrain Norepinephrine (NE) Levels in Convulsive and Nonconvulsive Rats after a Single Dose (78 µg/kg) of Soman

Time (hr)	Control Mean (ng/g tissue) ±S.E.M.	Convulsive Mean (ng/g tissue) ±S.E.M.	Non-Convulsive Mean (ng/g tissue) ±S.E.M.
1	349.1±8.2 (n=18)	165.2±18.5 (n=12; p<0.0001)	312.0±15.3 (n=15; p<0.05)
2	307.2±8.1 (n=9)	99.0±11.9 (n=9; p<0.0001)	305.9±37.0 (n=4; N.S.)
4	352.9±13.1 (n=10)	123.1±6.2 (n=8; p<0.0001)	354.5±16.7 (n=7; N.S.)
8	399.1±12.1 (n=9)	222.1±19.2 (n=6; p<0.0001)	378.4±11.6 (n=7; N.S.)
24	379.0±6.3 (n=14)	244.0±6.3 (n=11; p<0.0001)	365.2±20.8 (n=4; N.S.)
96	321.0±9.2 (n=9)	313.6±5.0 (n=5; N.S.)	343.1±15.3 (n=6; N.S.)

Significance levels are computed between indicated group and control group using two-tailed Student's t-test. N.S.: not significant.

Table 2

Olfactory Bulb Norepinephrine (NE) Levels in Convulsive and Nonconvulsive Rats following a Single Dose (78 µg/kg) of Soman

Time (hr)	Control Mean (ng/g tissue) ±S.E.M.	Convulsive Mean (ng/g tissue) ±S.E.M.	Nonconvulsive Mean (ng/g tissue) ±S.E.M.
1	197.1±9.5 (n=18)	123.7±12.5 (n=12; p<0.0001)	188.2±13.9 (n=15; N.S.)
2	208.8±4.8 (n=9)	90.2±5.9 (n=9; p<0.0001)	200.5±4.5 (n=4; N.S.)
4	211.4 ± 9.9 (n=10)	99.9 ± 3.7 (n=8; p<0.0001)	223.6±8.5 (n=7; N.S.)
8	226.6±13.4 (n=9)	155.7±11.2 (n=6; p<0.0001)	213.8±12.3 (n=7; N.S.)
24	205.7±5.5 (n=14)	159.7±6.9 (n=11; p<0.0001)	204.6±11.8 (n=4; N.S.)
96	207.5±6.8 (n=9)	192.8±3.6 (n=5; N.S.)	223.0±14.2 (n=6; N.S.)

Significance levels are computed between indicated group and control group using a two-tailed Student's t-test. N.S.: not significant.

Table 3

Mean Forebrain Norepinephrine (NE) Levels and Acetylcholinesterase (AChE) Activity in Convulsive and Nonconvulsive Rats after Soman Intoxication

	Control	Convulsive	Nonconvulsive
NE Level (ng/g tissue)	345.8 ± 6.2 (n = 11)	129.8 ± 5.4 (n = 10)	344.9 ± 13.3 (n = 7)
AChE Activity (IU/g tissue)	12.4 ± .31 (n = 11)	1.32 ± .38 (n = 10)	7.5 ± 1.24 (n = 7)

Values are mean ± SEM.

DISCUSSION

The present study demonstrates that (1) there is a strong relationship between forebrain NE depletion and soman-induced convulsions, (2) NE levels recover by 4 days in convulsive animals, and (3) NE depletion is a better predictor of the presence of convulsions than AChE inhibition. Despite the presence of other signs of intoxication, NE levels in nonconvulsive rats remained near control levels at all intervals tested. In contrast, the decrease in NE levels in convulsive animals was rapid and dramatic, with nearly 70% depletion from control levels in the forebrain at 2 and 4 hr after intoxication.

The strength of the correlation between convulsions and NE depletion is well illustrated in Figure 2, in which data for individual animals is presented. All convulsive rats had severely depressed levels of NE. NE levels in rats scored as nonconvulsive, on the other hand, cluster tightly around control levels. Further, the correlation between forebrain and olfactory bulb NE depletion suggests a global and not a localized depletion of NE.

Previous studies (Fosbraey *et al.*, 1990; Glisson *et al.*, 1974) did not examine the correlation between NE and the incidence of convulsions because, at the dose used in those studies, all animals convulsed. Moreover, in these earlier experiments, NE was measured only in the first 4 hr following OP intoxication. Thus, the duration of the soman-induced depletion of NE was not determined. In the present study, a dose of soman was used which caused seizures in only two thirds of intoxicated animals. NE and AChE levels could, therefore, be examined in relation to the presence or absence of convulsions. In addition, we monitored NE changes for 4 days after soman treatment in order to determine if NE levels recovered.

In agreement with previous studies (Glisson *et al.*, 1974; Fosbraey *et al.*, 1990), NE levels in our convulsive animals declined 50% within 1 hr and were minimum at 2 hr following soman injection. NE levels remained depressed 4 hr after intoxication and began to recover after 8 hr. The beginning of this recovery roughly coincides with the termination of convulsions. After 4 days, NE levels in convulsive animals were indistinguishable from control. Thus, in animals which survive convulsions, NE levels recover.

The same dose of soman produced widely different degrees of AChE inhibition (Figure 9). Presumably, variations in the rate of uptake into the circulation (depending on the exact placement of the injection), quantity of peripheral cholinesterases present in the individual animal, or access to the brain caused different proportions of the injected soman to reach the brain. The residual AChE levels in the brain do not, however, fall into distinct, non-overlapping, groups associated with convulsions as

NE levels do. Thus, the present results demonstrate a stronger association between NE depletion and convulsions than between AChE depletion and convulsions. These results, taken with recent studies of the pharmacology of LC neurons and of the postsynaptic actions of NE suggest that NE may play an important role in OP-induced convulsions.

Cholinergic Activation of NE Release. It is probable that the rapid decline in forebrain NE in convulsive animals is due to rapid synaptic release of NE from noradrenergic terminals. This possibility is consistent with recent electrophysiological results. *In vivo* extracellular and *in vitro* intracellular recording studies have demonstrated that LC neurons are strongly excited by cholinergic agonists (Engberg and Svensson, 1980; Egan and North, 1985, 1986; Adams and Foote, 1988). These studies suggest that cholinergic excitation of LC neurons following AChE inhibition could lead to a sustained release of NE from LC terminals.

Recent experiments in this laboratory confirm this inference. In anesthetized animals, systemic administrations of soman increase mean LC neuron firing rate by 5-fold (Ennis and Shipley, in press, Exp. Neurol.). Microinjections of soman directly into LC cause a similar increase in the firing rate of LC neurons. This increased firing rate continues for several hours. Systemic injection of scopolamine rapidly reverses the soman-induced firing rate increase in LC neurons (El-Etri *et al.*, 1990; Ennis and Shipley, in preparation). Thus, there is direct evidence that soman causes a sustained, probably muscarinic, excitation of LC neurons.

Thus, one effect of systemic soman injection is rapid, sustained, firing of LC neurons and increased release of NE. In convulsing animals, this period of rapid release may be followed by depressed NE release because the pool of stored transmitter is exhausted. The consequence of rapid, sustained NE release, or of NE depletion, for seizures depends on the effect of NE on seizure thresholds. There is conflicting evidence on the effect of NE on cortical excitability and susceptibility to seizures.

Effect of excess NE on seizure susceptibility

Evidence for Antiseizure Role for NE. Manipulations which increase NE levels in cortical structures by transplantation of NE neurons (Barry *et al.*, 1987) or by electrical stimulation of LC (Libet *et al.*, 1977) increase seizure thresholds. Forebrain NE depletion by 6-hydroxydopamine (6-OHDA) reduces seizure threshold (Mason and Corcoran, 1979). Such studies are inconclusive, however. Interpretation of the effects of electrical stimulation of LC is difficult because an electrode located in LC may unavoidably stimulate neurons in the adjacent parabrachial and lateral dorsal tegmental (LDT) nuclei. Both these structures contain neurons which project to the thalamus and cortex (Satoh and

Fibiger, 1986). LDT in particular may play a critical role in regulating cortical activity, as this nucleus projects heavily to the thalamic reticular nucleus which modulates thalamocortical excitability (Kayama *et al.*, 1986; Levey *et al.*, 1987; Steriade *et al.*, 1987). Thus, electrical stimulation of LC may activate other, non-NE neurons, which strongly regulate the excitability of cortical neurons. The interpretation of LC or forebrain NE fiber lesions is also complicated by time-dependent effects of 6-OHDA lesions (Lidbrink, 1974), which may be related to compensatory mechanisms acting to minimize the functional consequences of NE-specific lesions (Diaz *et al.*, 1978; U'Pritchard *et al.*, 1980; Berridge and Dunn, 1990).

Evidence for a Seizurogenic Role for NE. There is also evidence that NE *increases* susceptibility to seizures. In the single gene mutant mouse tottering (*tg/tg*), which is extremely susceptible to seizures, there is an increase of 50% in the number of LC fibers and NE in the forebrain (Levitt and Noebels, 1981). Recent *in vitro* cellular studies provide additional evidence that NE might lower seizure thresholds. NE disinhibits cortical neurons by mechanisms similar to those of ACh in hippocampal pyramidal neurons (Fain and Garcia-Sainz, 1980; Madison and Nicoll, 1982, 1986; Haas and Konnerth, 1983; Collins *et al.*, 1984; Gonzales and Crews, 1985; Gray and Johnston, 1987; Nicoll, 1988). This disinhibition should increase susceptibility to seizures. Consistent with this supposition, systemic administration of the ß-receptor *antagonist* propranolol reduces the duration of pentylenetetrazol-induced convulsions in rats (Louis *et al.*, 1982). In the hippocampal slice, ß-adrenergic *agonists* are proconvulsive (Mueller and Dunwiddie, 1983).

The results of the present study and other evidence reviewed above indicate that, following a convulsive dose of soman, there may be an excess not only of ACh, but also of NE in the forebrain. The existence of excessive levels of ***both*** ACh and NE is a unique circumstance which could have potent seizurogenic effects. Intracellular recording studies indicate that NE and ACh act upon neurons through the same second-messenger systems and ionic channels (Nicoll, 1988). Sustained excessive levels of NE and ACh might, therefore, act in synergistic fashion to produce disinhibitory actions much greater than those produced by either transmitter alone. Thus, it is possible that the combination of increased NE *and* ACh following soman intoxication could have potent seizurogenic actions.

<u>NE Pharmacology in the Treatment of OP Intoxication</u>. The a_2-agonist clonidine protects against OP-induced behavioral convulsions (but not lethality) as effectively as atropine. The protective effect of clonidine is synergistic with the protective effect of atropine (Aronstam *et al.*, 1986; Buccafusco and Aronstam, 1986; Buccafusco *et al.*, 1988). Presynaptic depression of ACh release by clonidine was proposed to explain this protective action of clonidine (Vizi, 1980; Aronstam *et al.*, 1986; Buccafusco and Aronstam, 1986; Buccafusco *et al.*, 1988). However, the direct excitatory

actions of soman on LC neurons (El-Etri *et al.*, 1990; Ennis and Shipley, in preparation) and the strong correlation between NE depletion and convulsions shown in the present study suggests a more direct mechanism for the anticonvulsive action of clonidine. It is well known that clonidine acts at a_2-adrenergic receptors on LC neurons to reduce their firing rate (Svensson *et al.*, 1975; Egan *et al.*, 1983; Marwaha and Aghajanian, 1982; Adams and Foote, 1988). Recently, clonidine was shown to block the excitatory action of ACh on LC neurons (Adams and Foote, 1988). Thus, the protective action of clonidine in soman-intoxicated animals may result from clonidine's ability to inhibit cholinergic activation of LC neurons as well as its presynaptic inhibition of ACh release from cholinergic terminals (Vizi, 1980).

Numerous agonists and antagonists specific to different classes of NE receptor are in clinical use. If NE release plays an important role in the response of the brain to OP intoxication, as suggested by the present and other studies, then adrenergic pharmacological intervention may prove useful in the treatment of OP poisoning.

6. CHOLINERGIC REGULATION OF TRANSMISSION IN THE COMMISSURAL CONNECTION BETWEEN THE OLFACTORY BULBS

Introduction

The nucleus of the diagonal band (NDB) is a part of the magnocellular basal forebrain system, which also includes projections from the medial septum to hippocampus and from the nucleus basalis to neocortex (Shute and Lewis, 1967; Divac, 1975; Moyano and Molina, 1982; Sofroniew, *et al.*, 1987; Broadwell, 1975; Broadwell and Jacobowitz, 1976; Mesulam, *et al.*, 1983). NDB innervates the ipsilateral main olfactory bulb (MOB), primary olfactory cortex, the parahippocampal region and the amygdala. Recent studies have revealed heterogeneity in transmitter content among these systems. In the progression from nucleus basalis, through medial septum to NDB, which represents a progression in target structures from neocortex to allocortex to paleocortex, there is a decreasing proportion of cholinergic neurons and an increasing proportion of GABAergic neurons (Brashear *et al.*, 1986; Rye *et al.*, 1984; Wainer *et al.*, 1985; Zaborszky *et al.*, 1986). In view of these differences in transmitter content, it is not clear whether the basal forebrain projections perform similar functions in the different target structures.

One function of the medial septal projection is regulation of transmitter release from the terminals of commissural and association axons in hippocampus (Yamamoto and Kawai, 1967; Hounsgaard, 1978; Ben-Ari, *et al.*, 1981; Valentino and Dingledine, 1981; Krnjevic, 1981; Rovira *et al.*, 1983). This presynaptic regulation appears to be mediated by acetylcholine (ACh). The anterior commissure is analogous to the hippocampal commissure and the neocortical commissural system, the corpus callosum. Part of AC connects the two main olfactory bulbs through the anterior wing of the anterior commissure (AC). These AC fibers originate in the anterior olfactory nucleus (AON) and terminate predominantly in the internal plexiform and granule cell layers of the contralateral olfactory bulb, where they synapse with granule cells. The centrifugal projection from NDB terminates to some degree in all portions of the olfactory bulb; however, there is a particularly heavy termination in the internal plexiform layer and the superficial half of the granule cell layer (Macrides *et al.*, 1981; Luskin and Price, 1983; Macrides and Davis, 1983).

Thus, a major part of the NDB projection to the bulb terminates in a location appropriate to contact commissural terminals. In view of the presynaptic inhibitory action of ACh in hippocampus, it is possible that one action of the NDB terminals in the olfactory bulb is regulation of transmitter release from AC terminals. Therefore, we sought to determine if the projection from NDB regulates synaptic transmission in the anterior wing of the anterior commissure and whether ACh mediates this regulation.

Methods

Surgical Preparation. Adult male Sprague-Dawley rats were anesthetized with chloral hydrate (400 mg/kg supplemented as needed) or with the gas anesthetic methoxyflurane (Metofane). Levels of both anesthetics were adjusted to maintain surgical anesthesia as judged by depth and rate of respiration and the presence of a moderate foot withdrawal reflex. Body temperature was maintained at 37°C with a heating pad. Anesthetized animals were placed in a stereotaxic apparatus tilted at a 30^0 angle around the rostral-caudal axis and the lateral aspect of the olfactory bulb and the lateral olfactory tract (LOT) were exposed by craniotomy after removing the contents of the orbit. The dorsal aspect of the skull was also exposed by a scalp incision.

Recording. Glass capillary pipettes, broken to approximately 5 micron tip diameter and filled with 1M NaCl, were used for most recording. DC preamplification was used in all experiments; the amplified signal was usually band-pass filtered between 0.1 Hz and 7.0 KHz. A recording electrode was inserted into the olfactory bulb before insertion of stimulation electrodes so that bulb responses could be used to guide placement of the stimulating electrodes. In all records presented here, the recording electrode was positioned in the granule cell layer (gcl). Data acquisition, display, and control of stimulus timing were done by computer (Motorola VME/10). Amplitudes of field potential responses were measured from the baseline to the peak of the negative (AC and PC) or positive (LOT) deflection.

Stimulation Electrodes. Initial stereotaxic coordinates were determined from the atlas of Paxinos and Watson (1986). Monopolar stimulating electrodes (000 insect pins insulated except at the tip) were lowered stereotaxically into the contralateral anterior wing of the anterior commissure (AC). Optimal placement was determined by observation of field potentials in the bulb generated by stimulation through this electrode. After the anterior commissure was located, the stimulating electrode was cemented to the skull using alpha-cyanoacrylate adhesive and dental acrylic. In some experiments a second electrode of similar construction was placed into the posterior part of the ipsilateral piriform cortex (PC) to stimulate ipsilateral afferents to the MOB. This electrode was also cemented to the skull after optimal placement was determined. A concentric bipolar stimulating electrode (0.25 mm outer diameter, 0.75 mm tip separation; Rhodes Medical Instruments, Woodland Hills, California), was guided stereotaxically into the NDB through a burr hole in the parietal bone.

A twisted bipolar stimulating electrode (100 µm wires, tip separation 300 µm) was placed against the lateral olfactory tract (LOT) under visual guidance. This electrode was used to antidromically activate axons of the mitral cells, the principal output neurons of the olfactory bulb. Antidromic activation of the mitral cells produces a well characterized field potential that undergoes a

change of polarity at the mitral cell body layer (mcl; Phillips, *et al.*, 1963). This transition and the presence of unit potentials were used to determine the location of the mcl. During experiments, the location of the recording electrode was always calculated from this marker.

All stimulating currents were delivered by a constant current stimulus isolation unit. Stimulating currents for the AC and PC electrodes were adjusted to two to three times threshold. With the monopolar stimulating electrodes used, this required 200 µA currents of 100 µsec duration. For the AC electrode located in the contralateral hemisphere, there is little danger of unintentional stimulation of structures projecting to the ipsilateral bulb. The stimulating electrode in posterior PC is likewise remote from other structures which project to MOB. Current spread could, however, cause antidromic activation of LOT, which runs along the surface of PC, or of LOT terminals in the superficial layer of PC. Antidromic LOT activation produces a field potential of opposite polarity to that of the PC association inputs to MOB. Thus, we adjusted stimulation electrode position and stimulating current parameters to produce a stable PC field potential without contamination from LOT activation. Stimulation of NDB required somewhat higher currents (300 µA, 300 µsec) because of the use of a bipolar stimulating electrode and the large extent of the nucleus. Issues related to selective stimulation of this nucleus have been discussed previously (Nickell and Shipley, 1988). After each experiment, sufficient current was passed through the electrodes to produce lesions at the stimulation site; the locations of the lesions were later determincd in histological sections.

In some experiments, drugs were applied directly to the exposed surface of the olfactory bulb. Soman (pinacolylmethylphosphonofluoridate; 1 mM) and atropine (10 mM) were dissolved in artificial cerebrospinal fluid (ACSF) and gently applied to the bulb surface with a 50 µl syringe. The drug solutions were removed by wicking away the solution and washing with ACSF. In the case of the irreversible cholinesterase inhibitor, soman, the extent of AChE inhibition at the termination of the experiment was determined by perfusing the animal with glutaraldehyde fixative and processing the tissue for AChE histochemistry (Van Ooteghem and Shipley, 1984).

Micro-Injection of Drugs. In other experiments, drugs were injected directly into the olfactory bulb from the recording pipette. Recording pipettes of approximately 10 micron tip diameter were filled with drug solutions (carbachol, acetylcholine) in 0.9% saline or ACSF and injected by short pressure pulses (Picospritz). Injected volumes were estimated by measuring the diameter of the droplet created in air by a known number of pressure pulses. The volume of solution injected by this method was, however, always somewhat uncertain because of the possibility of plugging of the pipette during insertion into the bulb. Thus, a method was developed which allowed direct measurement of injected volume and injection of an equal volume of vehicle into the same location.

Microbore pipettes (Fisher Scientific Company, Catalog Number 21-164-2A) were used. In these pipettes, ejected volumes of 60 nl result in a meniscus movement of 1 mm. To fabricate the double-barreled micropipette, one microbore tube was shortened and then bent in a flame. This shortened and bent tube was then cemented firmly to a second, straight microbore tube using epoxy cement. The straight, uncut, microbore tube was inserted into the chucks of a vertical pipette puller. The two tubes were then fused by heating them sufficiently to allow the chuck to be rotated 1/2 turn in each direction. After cooling, the fused tubes were pulled at a normal heat setting. The resulting double-barreled pipette was broken under a microscope to about 30 µm diameter. For recording evoked potentials, a length of formvar-insulated stainless steel wire (2 mil, 50 µm) was then brought into contact with the tip and fastened with a drop of cyanoacrylate adhesive. The wire was trimmed to extend about 200 µm past the end of the pipettes, leaving the tip uninsulated. Each barrel was filled from the back by applying suction while the pipette tip was immersed in the appropriate solution. Several minutes were often required for sufficient solution to be pulled into the shank of the pipette. Drug or vehicle solutions were ejected by short (20 msec) pressure pulses of 10 psi. Quantities of fluid ejected were measured by observing movement of the meniscus relative to a mm scale attached to the microbore tube.

Antidromic stimulation thresholds of AON terminals. Initial experiments suggested that inhibition of the AC field potential might be caused by presynaptic inhibition of AC terminals. Thus, we tested whether inhibition of the field potential was associated with changes in the antidromic activation threshold of AC terminals.

Terminals of AON neurons projecting to the ipsilateral bulb were activated using a bipolar stimulation and recording electrode constructed by gluing two 2 mil (50 µm) formvar-insulated stainless-steel wires side-by-side with 400 µm tip separation. The deeper of these electrodes was also used to record AC and NDB field potentials. A small switch located near the preparation allowed connection to the amplifier or stimulator without disturbance of the electrodes. The electrode assembly was advanced until the forward tip was about 100 µm below the mcl (as determined by observation of changes in the LOT waveform recorded simultaneously).

Single units in the contralateral AON were recorded using tungsten microelectrodes. AON units driven by stimulation of the ipsilateral bulb were tested for antidromic activation by three tests: 1) Constant latency, 2) ability to follow a train of 3 shocks at 3 msec interval, and 3) when the spontaneous firing rate was sufficient, the collision test. Unit activity was recorded on instrumentation tape; peristimulus time histogram (PSTH) records were constructed on-line using a computer.

Results

Field potential responses produced by AC stimulation. An extracellular electrode placed in the granule cell layer (gcl) of the olfactory bulb records a negative going field potential (Fig. 10A) in response to AC stimulation (Walsh, 1959; Nakashima *et al.*, 1978). The mechanisms responsible for this field potential have been well described; it is the result of synchronous postsynaptic excitatory currents flowing into the granule cells in the gcl (Walsh, 1959; Yamamoto *et al.*, 1963; Mori and Takagi, 1978). The extracellularly recorded AC-evoked field potential is, therefore, a good index of the total synaptic current entering the population of granule cells after AC stimulation. This index has been used by numerous authors in previous studies of synaptic activity in the olfactory bulb.

Micro-injection of cholinergic agonists into the olfactory bulb. To test for a cholinergic inhibition of this AC response, the cholinergic agonists carbamylcholine chloride (10 mM; n=5) or acetylcholine (1 mM; n=3) were locally injected into the lpl and gcl of the olfactory bulb from the recording pipette. Injections (estimated at 100-500 nl over 10 minutes) of either agonist consistently depressed the amplitude of the AC response (Figure 10B). The depression caused by ACh reversed after about 30 min, whereas the depression caused by carbachol did not reverse within the period of observation (> 1 hr). Saline or ACSF injections of similar volume produced only small and transient changes in AC response amplitude.

Because injection from the recording pipette did not allow precise measurement of the volumes injected, we conducted additional experiments using a calibrated double barreled pipette (Methods). In addition, since the AC synaptic response exhibits significant facilitation with repeated shocks (Mori and Takagi, 1978), we tested with trains of 4-10 AC shocks to determine if inhibition caused by carbachol injections could be reversed by homosynaptic facilitation.

Injections of 5 nmol carbachol solution (100 nl of 50 mM carbachol in saline or ACSF) caused a 50% decrease in the AC response. I.p. injection of 1 mg scopolamine, however, reversed the carbachol inhibition within 5 min. In the same preparations, equal or larger volumes of vehicle ejected from the second barrel caused only small changes in response amplitude (n=4). These changes are illustrated in Figure 11, which shows the amplitudes of the field potential responses to a train of 10 AC shocks, given at 100 msec intervals. As indicated in the figure legend, the data presented were recorded before and after a micro-injection of 200 nl saline, after micro-injection of 100 nl (5 nmol) carbachol, and after systemic injection of 1 mg scopolamine.

The total volume of vehicle or drug solution was injected over a period of 5 min. The decrease

in response amplitude caused by drug injection continued for an additional 10 minutes. With repeated shocks there is moderate facilitation of the AC response amplitude, but the response did not return to control amplitude.

Site of carbachol action. The late positive-going component of the field potential caused by stimulation of the lateral olfactory tract (LOT), like the AC potential, is the result of synaptic currents flowing into granule cells (Rall and Shepherd, 1968). Absence of changes in the LOT field potential after injection of carbachol would, therefore, be evidence that inhibition of the AC response is not caused by shunting or depolarization of the granule cells. To determine whether carbachol affected the LOT potential, the amplitude of the antidromic LOT field potential was monitored to determine whether carbachol injection also depressed the LOT potential.

Carbachol injections produced no measurable effect on the amplitude of the LOT potential (Figure 11B).

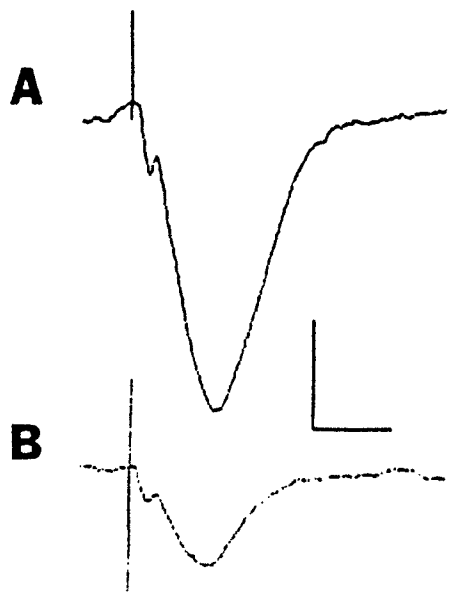


Figure 10. Normal Anterior Commissure (AC) Potential and its inhibition by Microinjection of Carbachol. A. Normal AC potential recorded by an electrode placed in the granule cell layer. B. AC potential inhibited by injection of an estimated 5 nmol (500 nl of 10 mM) carbachol. Calibration: 1 mV, 25 msec.

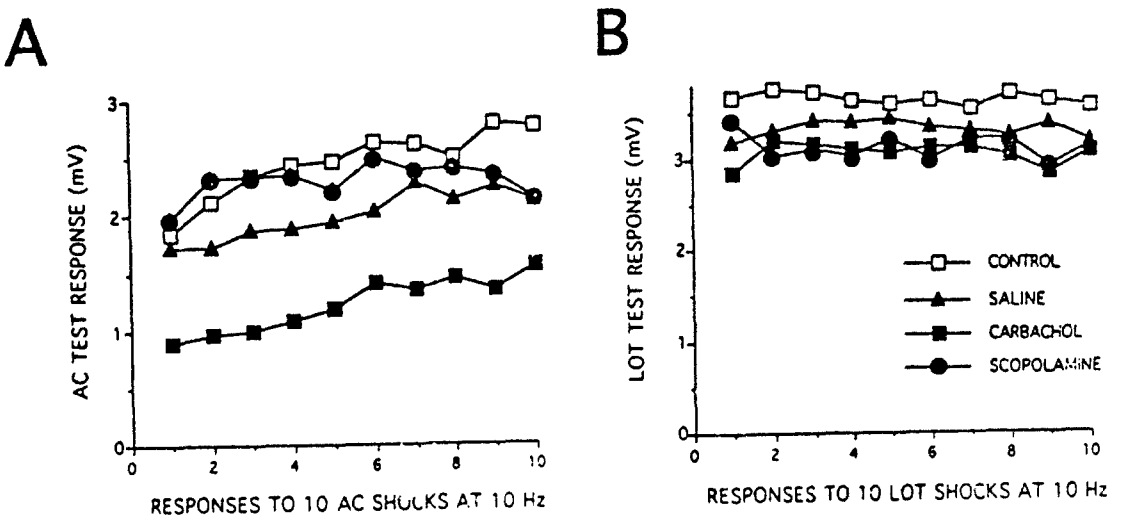


Figure 11. Effect of Saline and Carbachol Injection on Amplitude of Anterior Commissure (AC) and lateral olfactory tract (LOT) Responses. A. AC Responses. B. LOT Responses. A. Amplitudes of successive AC responses in a train of 10 shocks at 100 msec interval. B. Amplitudes of a similar train of shocks to LOT. As indicated in the legend, the plotted response amplitudes are before any injection, after injection of 200 nl of ACSF, after injection of 5 nM carbachol in 100 nl ACSF, and after ip injection of 1 mg scopolamine.

Effect of NDB stimulation and AChE blockade on AC response. The inhibition of the AC response by cholinergic agonists strongly suggested that endogenously released ACh inhibits synaptic transmission in the commissural pathway. If this is true, electrical stimulation of NDB should release ACh and reduce the AC response amplitude. Similarly, blockade of the degradative enzyme for ACh, AChE, might cause buildup of ACh tonically released from NDB terminals; this should also inhibit the AC response. AChE inhibition might also potentiate the cholinergic effects of NDB stimulation. Thus, we investigated the effects of NDB stimulation and of AChE inhibition on the amplitude of the AC response.

Single or double NDB shocks did not cause any measurable inhibition of the AC field potential (not illustrated). We, therefore, tested the effect of short trains of NDB shocks on the AC response amplitude (n=5). Typical results of these tests are shown in Figure 12 (A and B) and Figure 13. Figure 12B shows field potential responses to a train of four AC shocks beginning 500 msec after termination of 40 NDB shocks. As shown, the initial AC response is inhibited by the NDB stimulation, but the amplitude increases with repeated AC shocks so that the fourth shock is approximately the same amplitude as the control. Increasing the number of NDB shocks from 40 to 120 caused only a small additional inhibition of the AC response (Figure 13). In this figure the amplitudes of the first (A) and the fourth (B) AC responses are plotted. Because of facilitation of the AC response, the amplitude of the fourth AC response is always larger than the control amplitude of the first response. Thus, the inhibition caused by NDB stimulation can be partially overcome by facilitation of the AC pathway.

To determine the effect of AChE inhibition, we applied the irreversible cholinesterase inhibitor, soman, to the exposed surface of the olfactory bulb (1 mM in saline; see Methods). Because the enzyme inhibition caused by soman is irreversible, the region of the bulb in which AChE was inhibited could be determined by processing the tissue for AChE histochemistry after the experiment. Soman caused a well-defined zone of AChE inhibition, with complete inhibition on the lateral side of the bulb and normal AChE activity elsewhere (Figure 14).

In all cases (n=10) AChE inhibition decreased the AC response. The AC response amplitude decreased gradually for 20-30 minutes after application of soman, presumably reflecting the spread of AChE inhibition and accumulation of tonically released ACh. After AChE inhibition, NDB stimulation further reduced the amplitude of the AC potential. These effects are illustrated in Figures 12 (C and D) and Figure 13.

The reduction of the AC response by cholinesterase inhibition was reversed by topical application of 10 mM atropine in 3 of 5 cases tested (Fig. 13). In the remaining two cases there was no recovery of response. In the cases in which atropine reversed the effects of AChE inhibition, NDB

stimulation still inhibited the AC response (Figure 13). This residual inhibition may result from a noncholinergic component of the NDB projection or to failure of the topically applied atropine to block all muscarinic receptors.

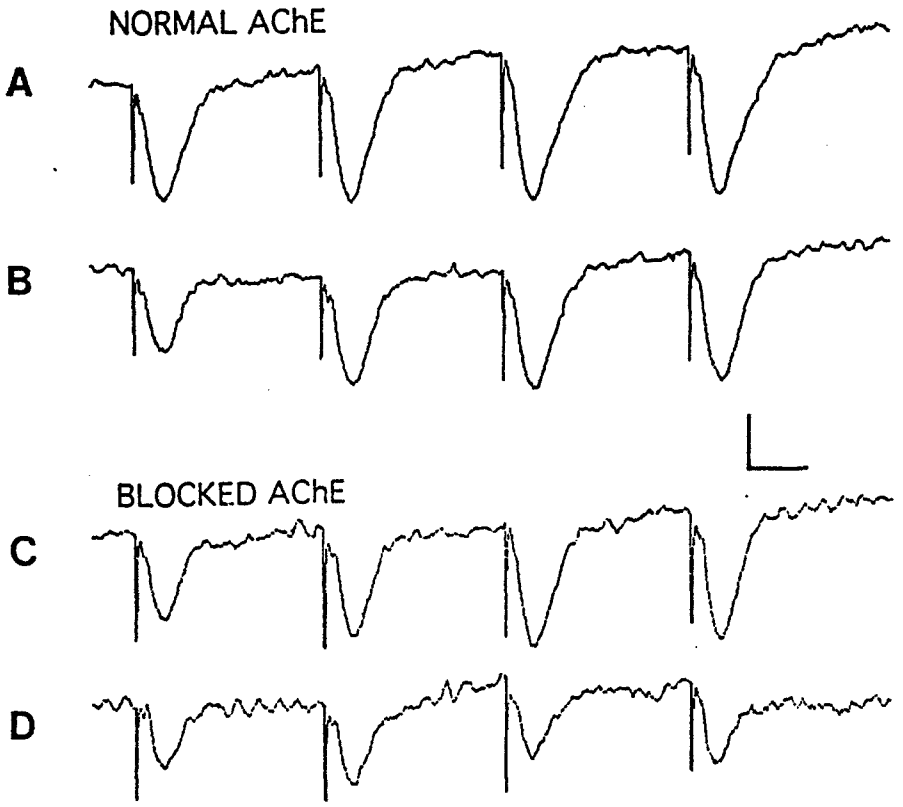


Figure 12. Effect of Nucleus of the Diagonal Band (NDB) stimulation on Anterior Commissure (AC) responses before and after Cholinesterase Inhibition. A, B: Normal AChE. C, D: AChE Blocked. A: Control AC responses. B: Responses to AC shocks beginning 500 msec after termination of a train of 40 NDB shocks at 10 Hz. C, D: Fifty minutes after topical application of soman. C: Initial response is suppressed but response amplitude increases with successive shocks. D. 500 msec following 40 NDB shocks at 10 Hz. Initial response was suppressed further, but there was little facilitation with repeated shocks. Oscillatory responses seen in these records are 50 Hz spontaneous activity often present after NDB stimulation. Calibration: 1 mV, 50 msec.

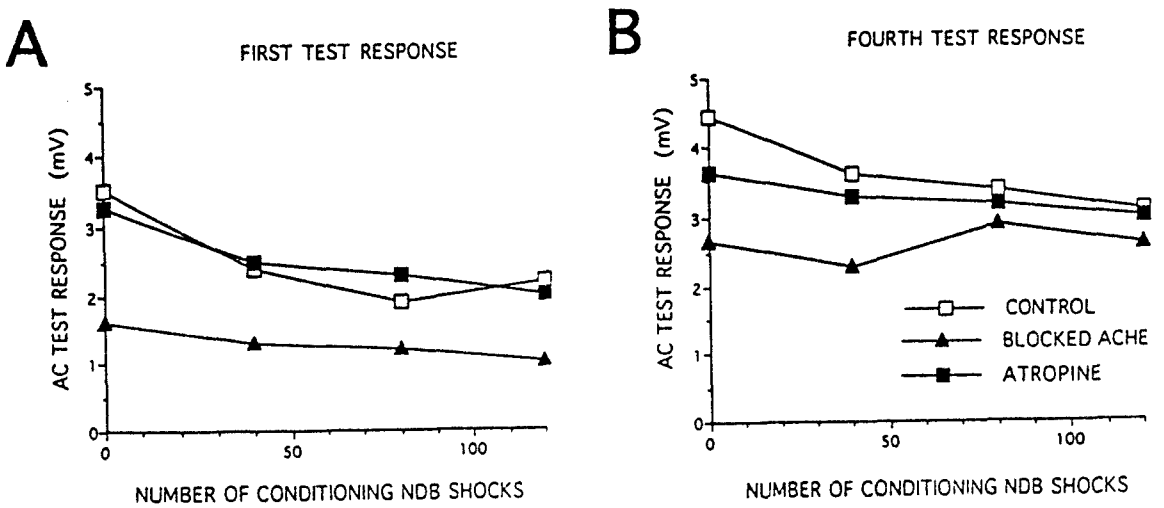


Figure 13. Amplitudes of Anterior Commissure (AC) response as a function of Nucleus of the Diagonal Band (NDB) shocks before and after cholinesterase Inhibition. A: Response amplitude to first shock in train of 4 AC shocks. B: Response amplitude to fourth shock in train of 4 AC shocks. As indicated by the legend, the plotted amplitudes are control, following cholinesterase inhibition by the irreversible AChE inhibitor soman, and following topical application of atropine.

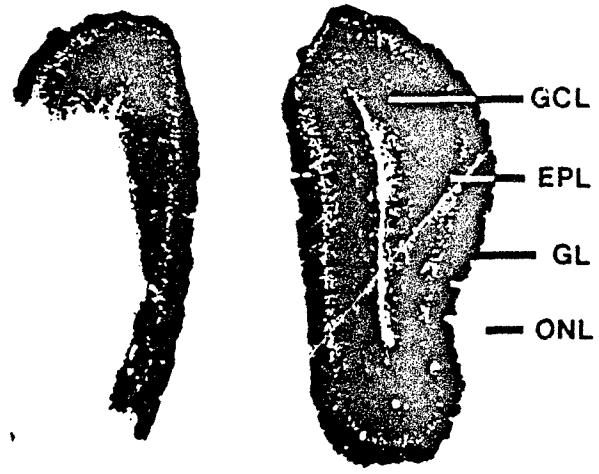


Figure 14. Acetylcholinesterase Inhibition following topical application of an Irreversible Cholinesterase Inhibitor. A 1 mM solution of soman in saline was applied to the exposed lateral surface of the main olfactory bulb. AChE inhibition advanced in a well-defined front with normal AChE activity on the far side of the front and complete AChE inhibition behind the front. The animal was perfused 1 hr following topical application of soman to the surface of the olfactory bulb.

Profound, long-term inhibition of AC response by longer trains of NDB shocks. We previously described the characteristics of the NDB-evoked field potential in MOB and its potentiation by 10 Hz stimulation (Nickell and Shipley, 1988). This stimulation rate is similar to the normal firing rate of NDB neurons and to the theta rhythm, which may be regulated by basal forebrain cholinergic neurons (Andersen *et al.*, 1979; Macrides *et al.*, 1982). Single NDB shocks produce a biphasic field potential recorded in the granule cell layer of the olfactory bulb. Stimulation for several seconds at 10 Hz causes a marked potentiation of this field potential, which decays to half amplitude within 2 seconds after termination of the stimulating train. Thus, 10 Hz NDB stimulation causes pronounced and relatively long-lasting changes in the state of the olfactory bulb circuitry that are not apparent with single, or short trains of NDB shocks.

In contrast with the moderate inhibition of the AC response caused by shorter trains of NDB shocks, trains of NDB stimulus shocks sufficient to cause potentiation of the NDB field potential caused a profound and long-lasting inhibition of the AC response (n = 20 animals). In some preparations, the AC response was completely suppressed for many seconds after NDB stimulation. Figure 15 shows the response to AC stimulation before and after NDB stimulation in two separate experiments. In both experiments, the initial AC response is completely suppressed at 10 (panel A) and 3 (panel B) seconds after a train of 160 NDB shocks. With repeated AC shocks, however, the AC response amplitude rapidly increases. In each preparation the response to a single AC shock would have been inhibited for many additional seconds in the absence of repeated AC stimulation. Thus, the increase in response amplitude seen in Figure 15 is caused by facilitation of the AC response and not by passive decay of the inhibition.

The duration of the inhibition is further illustrated in Figure 16, which shows the amplitude of responses to a single test shock at different intervals after NDB stimulation. A 10 minute rest period was allowed between each trial and a control AC response was obtained immediately before each period of NDB stimulation. The response returns to 50% amplitude after 11 seconds and is 75% of control after 30 seconds. In other preparations, the AC response was completely inhibited for a longer period, in some cases for 10 to 20 seconds (Figure 15B).

Other synaptic inputs to granule cells are not inhibited by NDB stimulation. The NDB stimulation that causes prolonged inhibition of the AC response produces a shorter-lasting potentiation of the NDB field potential. The LOT response is not significantly affected (Nickell and Shipley, 1988). Like the AC field potential the NDB and the LOT field potentials result from synaptic currents flowing into granule cells (Nickell and Shipley, 1988; Rall and Shepherd, 1968), although the LOT potential is the result of synaptic activity in the more superficial granule cell layer (gcl). Thus, the absence of

inhibition of NDB and LOT responses after NDB stimulation suggests that the NDB inhibition of the AC response is not the result of changes in granule cells. This was further tested by determining the effect of NDB stimulation on the field potential evoked in the olfactory bulb by stimulation of ipsilateral piriform cortex (PC). This projection terminates on granule cells in a pattern similar to the termination of the anterior commissure (Davis and Macrides, 1981; Luskin and Price, 1983). Thus, if the inhibition of the AC field potential is caused by depolarization or shunting of granule cells, then the PC potential should also be inhibited.

NDB stimulation sufficient to cause potentiation of the NDB field potential causes a similar potentiation of the PC field response (Figure 17A). Like the potentiated NDB field potential, the PC response returned to control amplitude within 2 seconds after the end of NDB stimulation (Figure 17B). In contrast with the potentiation of the PC response, the AC field potential was, as in previous experiments, inhibited at all times after NDB stimulation (Figure 17B). Identical results were obtained when the order of the AC and PC shocks was reversed. At longer periods after NDB stimulation both AC and PC responses were suppressed. Results similar to those illustrated in Figure 17 were obtained in 5 preparations.

Thus, PC responses, like LOT, and NDB field potential responses, are not affected, or increased, shortly after NDB stimulation, at the same time as the AC response is profoundly depressed. Since all these responses result from synaptic currents flowing into granule cells, it is improbable that the AC inhibition results from depolarization or shunting of the granule cells. This suggests therefore, that an alternative site of the inhibition of the AC response is a modification of the AC terminals. Facilitation of the inhibited AC response by repeated AC shocks (homosynaptic facilitation) is also consistent with changes in the AC presynaptic terminals. These considerations suggest that inhibition of the AC response is caused by reduced transmitter release from AC terminals rather than by post-synaptic mechanisms such as depolarization or shunting of the target granule cells. Thus, we tested directly for changes in AC terminals resulting from NDB stimulation.

Decreased excitability of AC terminals in the ipsilateral olfactory bulb after NDB stimulation. In spinal nerves, presynaptic inhibition of transmitter release is accompanied by depolarization and an increase in excitability of the inhibited terminals (Wall, 1958; Eccles *et al.*, 1961, 1962ab, 1963). Thus, we tested whether the decrease in the AC field potential amplitude after NDB stimulation is accompanied by a change in the excitability of AON terminals in the olfactory bulb.

A bipolar stimulating and recording electrode (Methods) was placed in the olfactory bulb with the leading tip located in the superficial part of the gcl 100-200 μm below the mitral cell layer. The

effect of NDB stimulation on the amplitude of the AC field potential was observed by recording through the leading tip of this bipolar electrode. NDB stimulation parameters were adjusted to produce the prolonged inhibition of the AC response illustrated in Figure 15.

Contralateral AON neurons activated by stimulation of the anterior commissure were isolated by slowly advancing an extracellular recording electrode through the AON while periodically stimulating through the AC electrode. Units driven by AC stimulation were tested for antidromic activation from the ipsilateral olfactory bulb electrode using the tests of constant latency, high-frequency following, and the collision test (Methods). Six of 12 antidromically activated units exhibited a latency of 3-6 msec; however, 6 other units which passed all three tests for antidromic activation had much longer latencies between 8 and 16 msec. These long-latency responses may represent neurons activated through small terminal branches of the AON axons.

The probability of activation of the isolated AON neuron was determined by constructing a peri-stimulus time histogram (PSTH) of the response to 20 bulb stimuli given at 300 msec intervals. So that changes in excitability could be detected, stimulus current through the bulb electrode was critically adjusted to be near threshold. Initial tests indicated that the excitability of AON terminals was *decreased* following NDB stimulation. No units were found in which the excitability was increased. In subsequent experiments, therefore, the stimulus strength applied to AC terminals was adjusted so that 18-20 of 20 stimulus shocks produced a spike in the isolated contralateral AON neuron.

Next, we tested for changes in the antidromic activation threshold of the isolated AON unit following NDB stimulation. Construction of the PSTH for antidromic activation of each AON unit began 500 msec after termination of the NDB stimulation. The threshold was considered changed only if the number of antidromic spikes decreased by at least 30% on each of 3 successive trials. Rest periods of 10 min were allowed between successive trials.

The effect of NDB stimulation was tested on the thresholds of 11 antidromically driven units in 5 animals. Of these 11 units, the excitability of 6 were decreased by NDB stimulation. This is illustrated in Figure 18, which shows a series of 3 pairs of PSTH records obtained before and after NDB stimulation. There is a consistent large decrease in the number of antidromic spikes following NDB stimulation. After NDB stimulation, most of the counted antidromic spikes occurred near the end of the 20 test shocks to AC, presumably because of decay of the inhibitory effect.

For each of the 6 neurons that passed the formal test for a change in activation threshold, a reduction of 50% or greater in the number of antidromic spikes could be obtained with critical

adjustment of the stimulus strength. The remaining 5 units were not consistently affected by NDB stimulation. There were no significant differences in the antidromic latencies of affected versus unaffected AON neurons.

Thus, the prolonged inhibition of the AC response caused by NDB stimulation is accompanied by significant changes in excitability of AC terminals in the bulb, although the direction of the change is opposite that of spinal afferents.

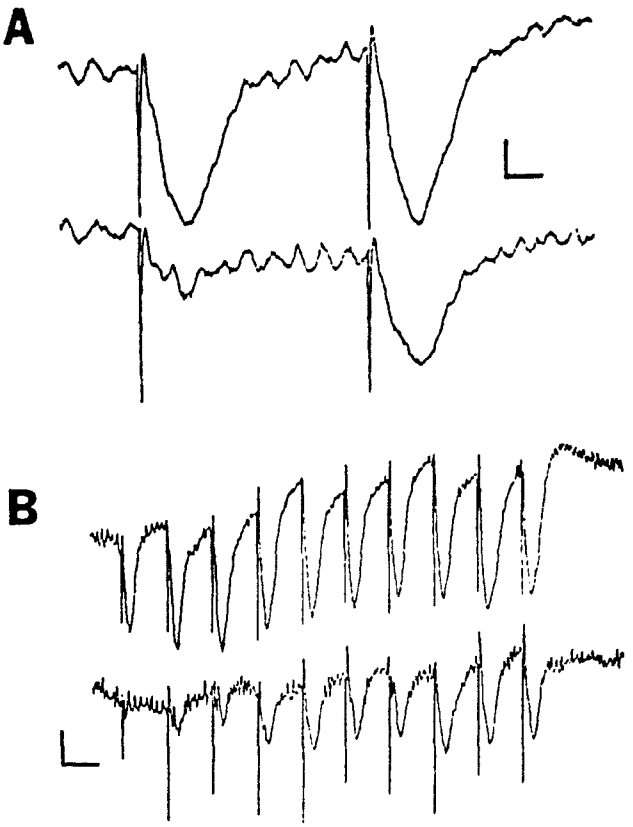


Figure 15. Inhibition of Anterior Commissure (AC) response by Nucleus of the Diagonal band (NDB) Stimulation. **A.** AC-evoked field potential responses three sec after the end of a train of 160 NDB shocks at 10 Hz. **B.** Different experiment, 10 sec after termination of 160 NDB shocks at 10 Hz. In both cases, the initial response is profoundly inhibited. However, with repeated shocks to AC the response returned to control values in a much shorter time than would have been required without facilitation. Oscillatory responses seen in these records are 50 Hz spontaneous activity often present after NDB stimulation. Calibration: 1.0 mV, 20 msec.

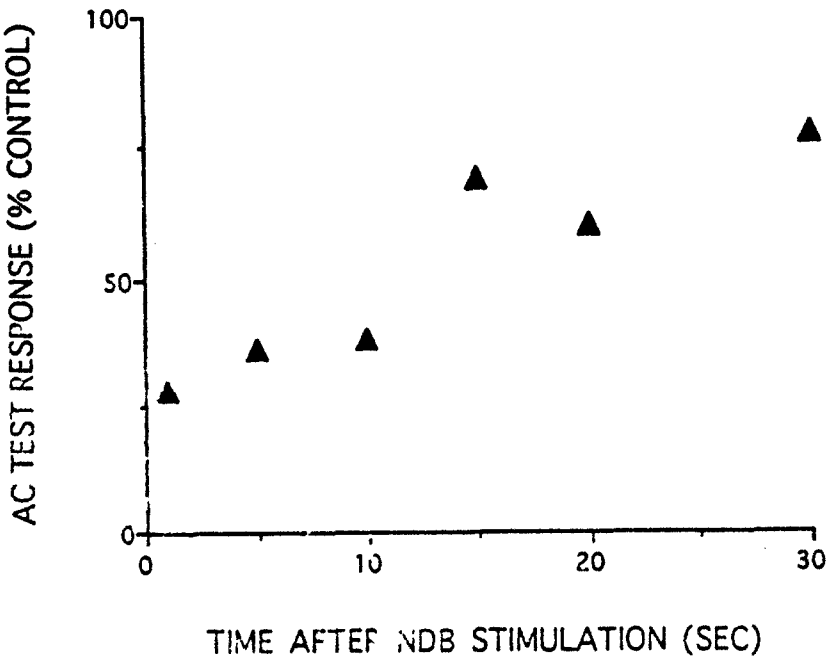


Figure 16. Time course of Anterior Commissure (AC) response inhibition by Nucleus of the Diagonal Band (NDB) stimulation. NDB was stimulated by 160 shocks at 10 Hz and AC was tested with a single shock to AC at the intervals indicated. A 10 minute rest period was allowed between trials.

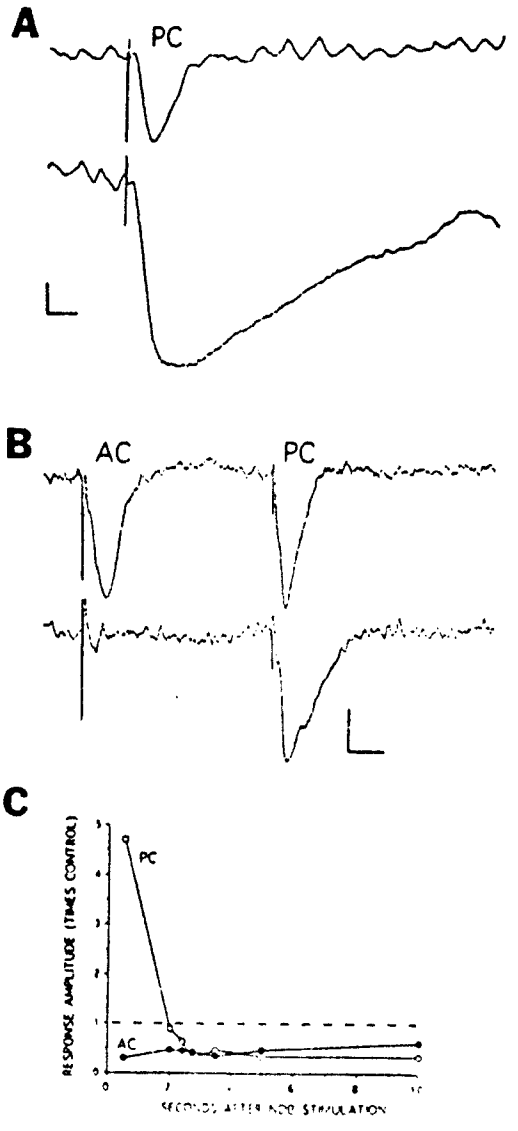


Figure 17. Differential effect of Nucleus of the Diagonal Band (NDB) stimulation on Commissural and Ipsilateral Association inputs to the Olfactory Bulb. A. One second after termination of train of 160 NDB shocks at 10 Hz. Top Record: Control. Bottom Record: One sec after termination of NDB stimulation. B. Comparison of PC and AC responses 2 sec after termination of NDB stimulation. AC response precedes PC response. Single shock to AC followed by single shock to PC. The AC response is still completely suppressed. Potentiation of the PC response was substantially decayed, but still larger than control. Calibration: 1.0 mV, 50 msec.

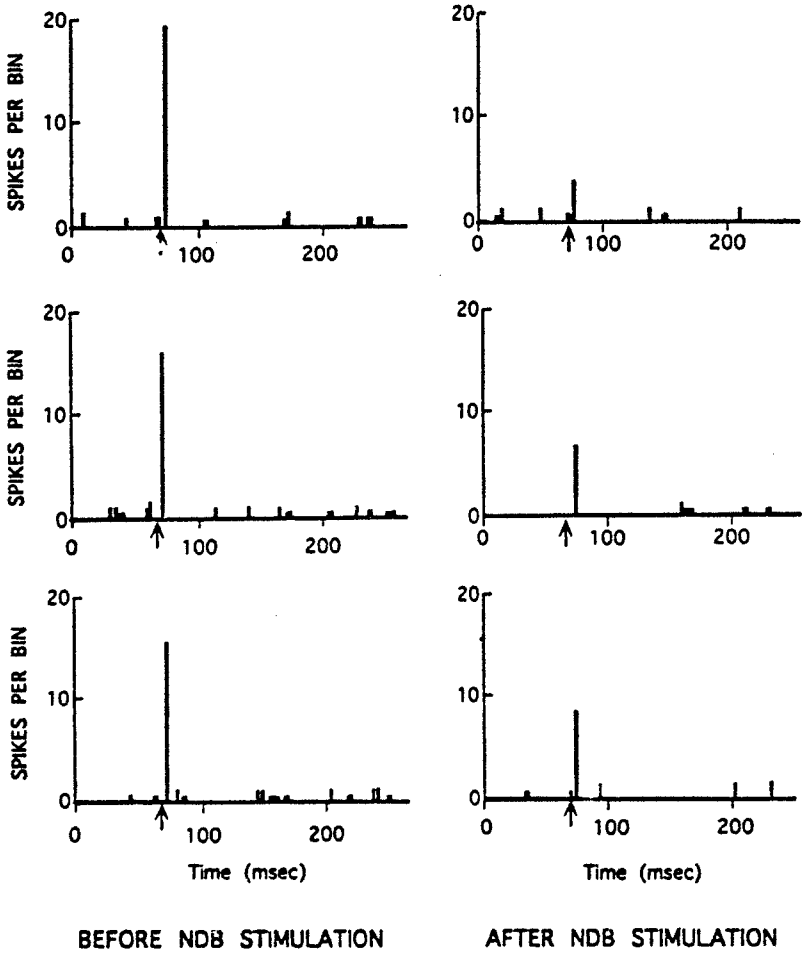


Figure 18. Decreased excitability of Anterior Olfactory Nucleus (AON) terminals following Nucleus of the Diagonal Band (NDB) stimulation. Left, before, and right, after, 200 NDB shocks at 10 Hz. Each PSTH record is the cumulative response of a neuron recorded in the AON to 20 shocks to the right olfactory bulb. Time of shock shown by arrow. At the stimulus strength used, this AON unit was antidromically activated by 17/20 olfactory bulb stimuli with a latency of 3.7 msec. The AON unit could also be antidromically driven by stimulation of the anterior commissure. Trials were separated by 8 min rests between NDB stimulation periods.

Discussion

The results of the present experiments can be summarized as follows: 1) Exogenous application of cholinergic agonists reduces the amplitude of field potentials caused by electrical stimulation of AC. 2) Blockade of the ACh degradative enzyme AChE caused a similar depression of the AC response. 3) Both these effects were blocked by muscarinic antagonists. 4) Stimulation of magnocellular basal forebrain neurons, which are the source of cholinergic input to the olfactory bulb, also inhibited the AC response. 5) The inhibition is selective to the AC response, since the responses evoked by stimulation of other afferents to the olfactory bulb that synapse with granule cells were not inhibited by NDB stimulation. 5) The inhibition of the AC response was prolonged but could be rapidly reversed by homosynaptic facilitation. 6) The excitability of some AC terminals in the olfactory bulb was significantly decreased by NDB stimulation.

Transmitters in the NDB projection. At least 25% of the NDB neurons projecting to the olfactory bulb are cholinergic (Brashear *et al.*, 1986; Rye *et al.*, 1984; Wainer *et al.*, 1985; Zaborszky *et al.*, 1986). The observed inhibition of the AC response after AChE inhibition in the olfactory bulb is, therefore, presumably due to buildup of ACh tonically released from these cholinergic terminals. Microinjection of cholinergic agonists into the vicinity of AC terminals in the bulb mimicked both the effect of AChE inhibition and NDB stimulation. The reversal of the inhibitory effects of both cholinergic agonists and AChE inhibition by muscarinic antagonists further supports the cholinergic nature of the inhibition and demonstrates the involvement of muscarinic receptors. Many other NDB neurons projecting to the olfactory bulb contain GABA (Brashear *et al.*, 1986; Rye *et al.*, 1984; Wainer *et al.*, 1985; Zaborszky *et al.*, 1986) and a peptide transmitter (galanin) has been colocalized with ACh in some NDB neurons (Melander *et al.*, 1985). Electrical stimulation in NDB probably releases these transmitters and possibly others not yet identified. Thus, ACh, acting at muscarinic receptors, certainly contributes to inhibition of the AC response by NDB stimulation but other transmitters and receptors may also be involved.

Presynaptic locus of the inhibition. The inhibition of the AC response by cholinergic agonists and by NDB stimulation is probably due to decreased transmitter release from AC terminals, i.e. presynaptic inhibition. The evidence for this interpretation is as follows:

First, other synaptic inputs to granule cells are not similarly affected by NDB stimulation, cholinergic agonists, or AChE inhibition. The LOT potential, which is generated by currents flowing into granule cells from synapses located in the external plexiform and granule cell layers, was not affected by any of the manipulations used in the present experiments. The ipsilateral PC field

potentials, which are generated by synaptic currents flowing into granule cells in the gcl, were strongly potentiated at short times after NDB stimulation, when the AC response was completely suppressed. The termination pattern of the ipsilateral PC projection in the bulb is similar to that of AC (Davis and Macrides, 1981; Luskin and Price, 1983; Schoenfeld and Macrides, 1984). Therefore, unless NDB selectively terminates upon only those granule cell processes that also receive AC terminal input, the inhibition of the AC response cannot be the result of depolarization or shunting of granule cell membranes.

Second, the decreased excitability of AC terminals in the ipsilateral bulb following NDB stimulation is direct evidence for a presynaptic locus of the inhibition. In other systems, several presynaptic mechanisms have been implicated in modulation of transmitter release. Stimulation of spinal roots is followed by reduced transmitter release, depolarization of the nerve trunk, and increased excitability of terminals (Wall, 1958; Eccles *et al.*, 1961, 1962ab, 1963). Hounsgaard (1978) found increased terminal excitability following application of ACh to afferent fibers in the hippocampal slice. Presynaptic inhibition of glutamate release from crustacean excitatory motor axons is mediated by increased Cl^- conductance (Takeuchi and Takeuchi, 1966). There is also evidence for increased K^+ conductance or blockage of voltage gated Ca^{++} channels as mediators of presynaptic inhibition (Dolphin and Scott, 1987; Bean, 1989). Any of these mechanisms might change the activation threshold of terminals.

The decreased excitability of AC terminals in the bulb contrasts with the increased excitability observed in spinal roots and hippocampus. This may indicate that the presynaptic inhibition described here is mediated by a different mechanism than mediates presynaptic inhibition in the hippocampus and spinal cord. Either an increase in Cl^- conductance or a decrease in the voltage sensitive Ca^{++} conductance might reduce terminal excitability. However, regardless of the mechanism, the fact that terminal excitability *changes* following NDB stimulation indicates that there is a direct effect of NDB stimulation on these terminals. This, together with other the other characteristics of the inhibition of the AC response discussed above, suggests a presynaptic locus for the effect.

Comparison with analogous systems. The NDB projection to the olfactory bulb is analogous to the medial septal projection to the hippocampus. Both are part of the basal forebrain system which projects to cortical structures; both are partially cholinergic, although the medial septal projection contains a larger proportion of cholinergic fibers (Brashear *et al.*, 1986; Rye *et al.*, 1984; Wainer *et al.*, 1985; Zaborszky *et al.*, 1986). It is, therefore, of interest to compare present findings with previous studies of the medial septal-hippocampal system. As in the olfactory bulb, exogenous application of cholinergic agonists reduces transmitter release from terminals of commissural afferents in

hippocampus (Yamamoto and Kawai, 1967; Hounsgaard, 1978; Ben-Ari, *et al.*, 1981; Krnjevic *et al.*, 1981; Valentino and Dingledine, 1981; Krnjevic and Ropert, 1982; Rovira *et al.*, 1983). Medial septal stimulation, however, increases the response of hippocampal granule cells to afferent inputs (Krnjevic and Ropert, 1982). The present study is apparently the first to demonstrate an inhibition of commissural terminals by endogenously released transmitters from magnocellular basal forebrain neurons.

A partial explanation for the difference between the effects of medial septum and NDB stimulation is the excitatory effects of exogenously applied ACh on pyramidal cells (Ben-Ari *et al.*, 1981; Krnjevic *et al.*, 1981; Krnjevic and Ropert, 1982; Cole and Nicoll, 1984). The mitral cells of the olfactory bulb, however, are not directly excited by cholinergic agonists or by NDB stimulation (McLennan, 1971, Nickell and Shipley, 1988). Therefore, the inhibitory actions of NDB stimulation on commissural terminals in the bulb may be manifest because it is not masked by excitation of mitral cells, whereas with electrical stimulation of the medial septum the excitatory actions of ACh on the pyramidal cells mask the inhibitory actions of ACh on commissural and association inputs.

Inhibition of afferent terminals may be a common feature of basal forebrain projections to cortical structures, although the proportion of the inhibition resulting from cholinergic receptors may differ. It is possible that the characteristics of the inhibition of the anterior commissure terminals following NDB stimulation are similar to those which would be observed in commissural inputs to the hippocampus if the excitatory effects of septal stimulation on pyramidal cells were removed.

Functional significance. Transection of the anterior wing of the anterior commissure reduces olfactory acuity (Bennett, 1968). The profound AC response inhibition produced by NDB stimulation might be similar to a transection of the commissure: sufficient activation of NDB would effectively uncouple the two olfactory bulbs. The ability of repeated stimulation to restore the response amplitude, however, suggests that the function of the inhibition may be to block the effects of isolated or short trains of spikes in AC axons, while allowing the longer trains to exert maximal effect. This action might prevent random activity in one bulb from affecting the contralateral bulb, while allowing effective transfer of the more ordered activity that might result from detection of an odor.

Part of the anterior commissure is sufficient for retrieval of olfactory memories from the opposite hemisphere (Teitelbaum, 1971; Kucharski and Hall, 1987; Kucharski *et al.*, 1986). Disruption of the cholinergic regulation of this commissural connection, as probably occurs during Alzheimer's Disease, might therefore be expected to interfere with memory functions involving transfer of information between the two sides of the brain.

7. CONCLUSION: A TWO-STAGE MECHANISM FOR SOMAN-INDUCED SEIZURES

The results presented above provide a new picture of the events leading to induction of seizures by organophosphates. The sensitive probe for cellular stress, *c-fos*, is generated in two structures very soon after soman intoxication. The first of these, PC, receives a very heavy cholinergic innervation from the basal forebrain and is the structure most susceptible to gross damage after soman exposure. Stimulation of the known source of the cholinergic input to the PC, the horizontal limb of the diagonal band, resulted in the same pattern of *c-fos* expression in the ipsilateral PC as that caused by systemic soman. Thus, it appears that *c-fos* may be an early marker for seizure-induced injury and that cholinergic hyperstimulation is causal to seizures.

The second structure expressing *c-fos* soon after soman intoxication is the LC, the source of noradrenergic innervation of the forebrain. Studies from other laboratories had demonstrated that cholinergic agonists stimulate LC neurons. Thus, it was probable that the *c-fos* in LC following soman intoxication reflected excessive activity of LC neurons caused by build up of ACh after destruction of the degradative enzyme AChE by soman. We tested this hypothesis directly by microinjecting soman into LC while recording from single-locus cells. As predicted, injection of soman caused LC neurons to increase their firing rate far above their normal physiological range. This increased activity was sustained for as long as 2 hr, the longest period examined. This increased firing probably accounts for the earlier observation that NE levels in forebrain are decreased by soman intoxication.

Taken together, these findings support a new hypothesis for the generation and maintenance of seizures by soman (Shipley *et al.*, 1988). This hypothesis postulates that there are two stages in the generation of seizures by soman intoxication. In the first stage, cortical neurons, most important in the PC, are subjected to excess ACh as a result of cholinesterase blockade. But, this direct effect of soman intoxication is accompanied by an excess of NE, which is secondary to accumulation of ACh in LC. Studies in other central neurons, primarily the hippocampal pyramidal cell, have shown that both NE and ACh can act through similar mechanisms to reduce the effectiveness of inhibitory conductances which would otherwise prevent the neuron from sustaining long trains of discharges. These conductances are activated by membrane depolarization or by the calcium ions which enter the cell after an action potential and block further depolarization and spiking. Both NE and ACh modulate the strength of these inhibitory currents through similar second-messenger systems (Nicoll, 1988). The coupling of the two transmitters to the same effector systems suggests the possibility that there could be a synergistic (more than additive) interaction when both transmitters are present (Shipley *et al.*, 1988). Even without this postulated synergism, however, it is clear that simultaneous excess of NE and ACh could lead to greater excitation than is possible with either transmitter alone and that this simultaneous

excess of NE and ACh could be a necessary condition for generation of seizures in the target cortex.

NE levels drop rapidly in seizing, but not in nonseizing, soman-intoxicated rats. This observation is compatible with at least two scenarios for the involvement of NE in the generation of convulsions:

1. Depletion of NE depends on the degree of AChE inhibition in LC. In those animals in which AChE inhibition exceeds some threshold, LC neurons may fire at a rate which results in release of NE beyond the ability of the cell to synthesize the transmitter. The rapid release of NE might be the trigger necessary for generation of convulsions in these animals. In animals with slightly more residual AChE activity, regulatory processes within LC might maintain LC neuron firing rates at a physiologically sustainable level; this lower rate of NE release might be insufficient to drive target neurons into convulsive activity. The difference between convulsing and nonconvulsing rats might then reflect the amount of AChE in one critical region of the brain.

2. NE might be decreased in seizing but not nonseizing rats because of secondary effects of the convulsions such as anoxia or buildup of toxic substances (Flynn and Wecker, 1986). In this case, NE might be rapidly released in both convulsive and nonconvulsive rats. NE might be essential to the generation of seizures, but differences in NE release would not be the trigger.

Two observations suggest that NE decreases are not the result of general effects of seizures. First, NE levels in guinea pig cortex and hippocampus are decreased at 15 and 30 min after soman intoxication, before the beginning of convulsions (Fosbraey *et al*, 1990). Second, other monoamines are not changed or have increased turnover following OP intoxication, suggesting that monoamine synthetic pathways are not impaired (El-Etri *et al*, in preparation; Fosbraey *et al*, 1990; Glisson *et al*, 1974).

The present data provide no direct measure of the rate of release of NE. However, the rapid decline of NE levels in convulsing animals strongly suggests rapid release of the transmitter. The decrease in total NE presumably reflects exhaustion of stored NE by rapid release. After exhaustion of stored transmitter, release would be limited by the maximum rate of neosynthesis. Thus, it is probable that soman intoxication results in an initial surge in NE release. There is no available evidence on the rate of release after this initial surge.

Once seizures are triggered, however, both NE and ACh might become unnecessary for their maintenance. It has long been known that prolonged electrical or chemical stimulation of cortical

inputs can cause permanent convulsive activity. One possible explanation for these observations is the phenomenon of long-term potentiation (LTP), in which tetanic stimulation of excitatory amino acid (EAA) inputs to a cortical structure results in permanent strengthening of synaptic connections. A particular class of EAA receptor (NMDA receptors) is required for generation of LTP, and pharmacological blockade of this receptor can block both LTP and seizures (Milan *et al.*, 1986, 1988; Braitman and Sparenborg, 1989; Olney *et al.*, 1990; Shih *et al.*, 1990; Switzer *et al.*, 1990).

Thus, there may be two stages in the generation of soman-induced seizures (Shipley *et al.*, 1990).

1. Initial induction. In the first stage, excess ACh and (as argued here) NE result in removal of normal regulatory processes from neurons in PC. As a result, cortical neurons increase their firing rate, therefore increasing the amount of EAA they themselves release onto other cortical neurons. Mutually excitatory reciprocal interactions between cortical neurons then lead to positive feedback of stimulation, activation of NMDA receptors, and seizures.

2. Maintained expression. Once initiated, the rapid firing of these neurons associated with convulsions might cause strengthening of synaptic connections; eventually, relatively little excess ACh and NE might be needed to sustain the convulsions. Thus, in the second stage, seizures might become independent of cholinergic or adrenergic mechanisms.

These hypotheses have important therapeutic implications:

1. Involvement of NE in generation of seizures. If excess norepinephrine is necessary for soman induced seizures, then the well-developed pharmacology of adrenergic receptors may provide promising therapeutic approaches. It has already been shown that the a_2-agonist clonidine provides significant protection against soman-induced convulsions (Buccafusco and Aronstam, 1986; Buccafusco *et al.*, 1988). Adams and Foote (1988) have shown that clonidine blocks the excitatory action of carbachol on LC neurons. A likely basis of this effect is the ability of this agonist to inhibit firing of LC neurons by stimulation of autoreceptors on LC neurons (Egan *et al.*, 1983; Marwaha and Aghajanian, 1982). It is probable that by combining clonidine with antagonists of postsynaptic adrenergic receptors and cholinergic antagonists, soman-generated seizures could be prevented without the use of barbiturates of excitatory amino acid antagonists.

The use adrenergic agents which have been in clinical use for many years to prevent seizures and resulting brain damage might have significant advantages over the use of NMDA antagonists to

suppress seizures. The NMDA antagonists are relatively untested clinically, may increase brain injury (Olney *et al.*, 1989), and, at doses necessary to prevent seizures, may cause psychological disturbances. Thus, if effective, adrenergic agents such as clonidine offer an attractive alternative therapy.

2. Independence of seizures and ACh after induction of convulsions. The possibility that, after some threshold is passed, convulsions may become independent of cholinergic or adrenergic mechanisms suggests that timing of treatment is critical. During induction of seizures, treatment with cholinergic blockers and adrenergic drugs may block seizures; however, once these seizures have been maintained for some undetermined time, these agents may be ineffective, and sedatives or EAA antagonists may be necessary.

3. Importance of early treatment. Our finding of *c-fos* expression at very short times after soman administration may suggest that some damage is occurring to brain structures even before the onset of behavioral convulsions and long before gross brain damage would be visible in histological sections. Thus, immediate treatment of exposed personnel is imperative.

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